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Stanford University	May 14, 1952

Electron Microscopy of Leptospiral Strains. (19564)

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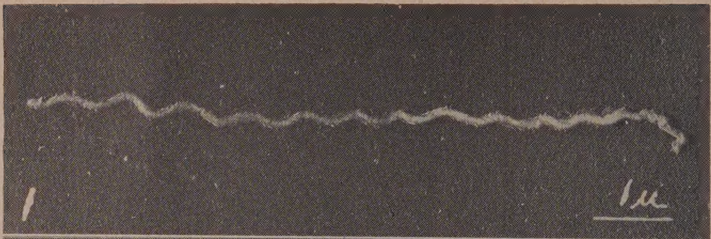
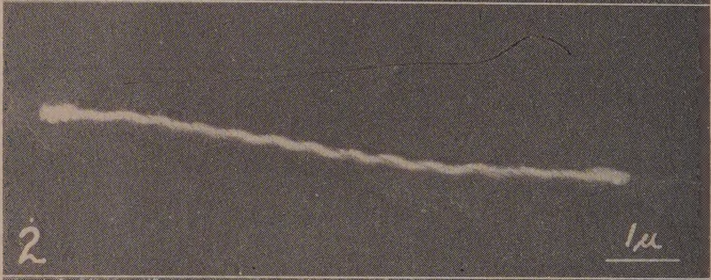
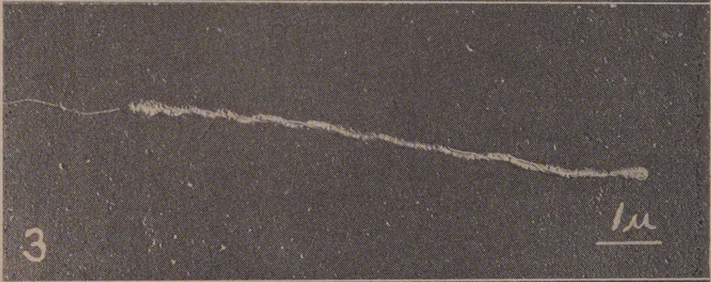
Although several studies(1,2) have been made on the morphology of leptospirae, additional information about structural details of these organisms is desirable. The following strains of leptospirae currently under study in this laboratory were therefore prepared for electron microscopy: *L. icterohemorrhagiae,* Wijnberg obtained from the University of Tropical Medicine, Leiden, The Netherlands; *L. autumnalis,* Akiyami A, *L. bataviae,* Swart van Tienen, *L. pomona,* and *L. mitis,* Johnson obtained from the National Institutes of Health, Bethesda, Maryland; *L. hyos* obtained from the Malbran Bacteriological Institute, Buenos Aires, Argentina; *L. bovis,* Palestine obtained from the Hebrew University, Jerusalem, Israel; and *L. canicola* isolated at the Veterinary Division, Army Medical Service Graduate School, Washington, D. C.

Materials and methods. Five-day-old cultures of leptospirae grown in Schüffner's modification of Verwoort's medium(3) were employed. Cultures were centrifuged at 20,000 g in the International PR-1 refrigerated centrifuge for 10 minutes at 5°C. The supernatant fluid was discarded and the leptospirae washed in M/15 phosphate buffered







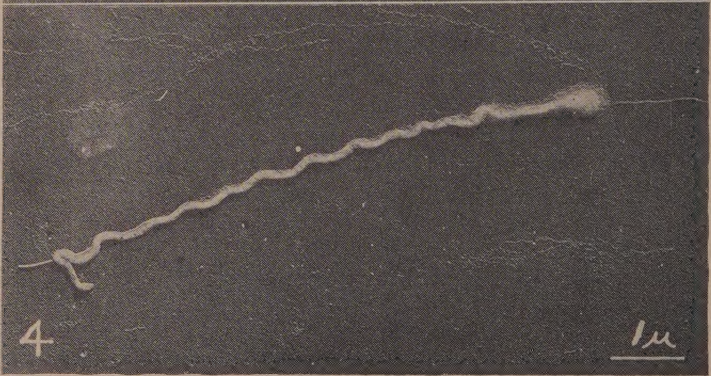
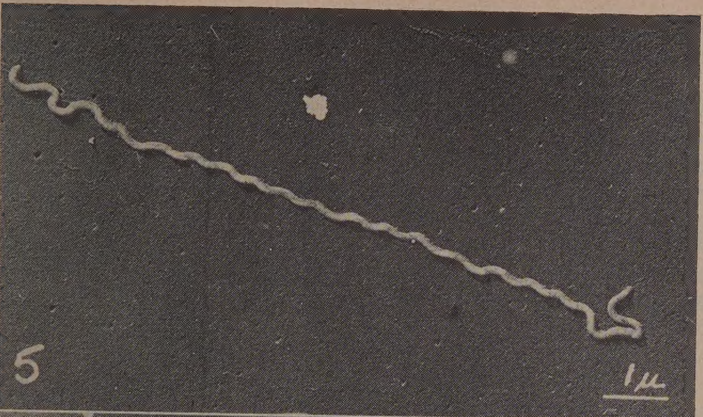
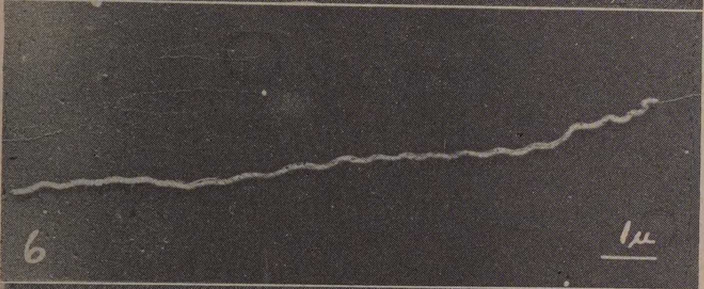


FIG. 1. *L. autumnalis,* Akiyami A, chromium shadowed, magnification 10,750 ×.
FIG. 2. *L. autumnalis,* Akiyami A, " " " 10,750 ×.
FIG. 3. *L. hyos,* chromium shadowed, magnification 9,200 ×.
FIG. 4. *L. hyos,* " " " 10,600 ×.

saline at pH 7.0. The suspensions were centrifuged again at 20,000 g for 10 minutes and the supernate discarded. Final suspension was made in the same buffered saline to approximately half the original volume. A drop of each of the freshly prepared suspensions was placed on standard stainless steel specimen grids which had been previously coated with a parlodian membrane. Excess solution was removed with a micropipette and the





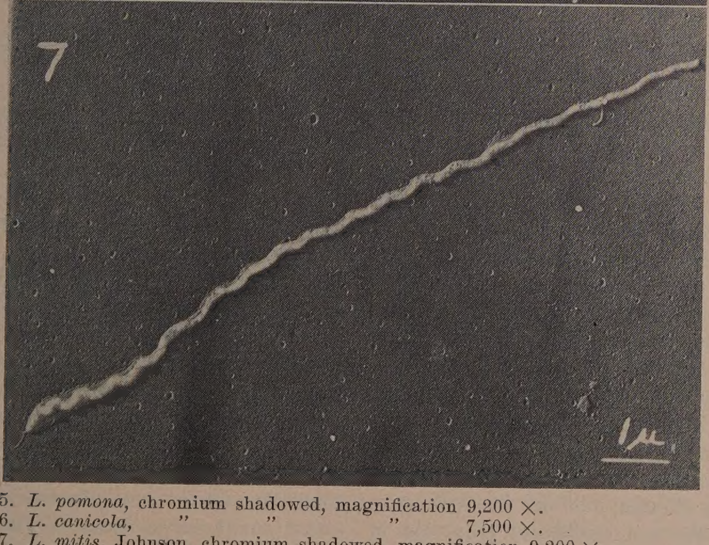


FIG. 5. *L. pomona,* chromium shadowed, magnification 9,200 ×.
FIG. 6. *L. canicola,* " " " 7,500 ×.
FIG. 7. *L. mitis,* Johnson, chromium shadowed, magnification 9,200 ×.

specimens dried in air. The screens were shadowed with 23 mg of chromium at an angle of 11°. An RCA model EMU-2B electron microscope was used. Specimens of each strain were examined and representative organisms photographed.

Results. Leptospirae were evenly distributed on the screens. Extended rather than curled specimens were chosen for electron photomicrography since they more clearly revealed morphological details.

The extremely short shadows indicate that the organisms were flattened by the drying process. Considerable variation in length was seen in all strains, ranging from 6 to 15 μ, occasional specimens being too long to be included in a single microscope field. General morphological features were common to all strains, such as a spiral, or cork screw, pattern. Shadowing revealed a fine filament entwined about the main structure of the organisms which often extended beyond one or both ends of the leptospirae.

The accompanying figures are typical electron photomicrographs of *L. autumnalis*, Akiyama A, *L. hyos*, *L. pomona*, *L. canicola*, and *L. mitis*, Johnson. Electron photomicrographs of *L. bataviae*, Swart van Tienen, *L. bovis*, Palestine, and *L. icterohemorrhagiae*, Wijnberg showed organisms that were indistinguishable morphologically from those illustrated in the figures.

Discussion. Length and diameter observed agree with the findings of Morton and Anderson(1). Shadowing with chromium precluded study of internal structure. In contrast to the observations of Morton and Anderson(1) and van Thiel and van Iterson(2) an axial filament entwined uniformly along the central cell mass was demonstrated in each leptospiral strain examined.

Summary. Eight strains of leptospirae were examined by electron microscopy. No morphological differences were found between strains. All strains possessed an axial filament. Diameter of the strains was approximately 0.1 μ, lengths varied from 6 to more than 15 μ.

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Influence of Cortisone on Experimental Hypersensitivity and Circulating Antibody in the Guinea Pig. (19565)

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(Introduced by Samuel Bukantz.)

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Previous studies(1,2) have demonstrated that cortisone suppressed the Arthus reaction and reduced the concentration of serum antibody in rabbits sensitized with egg albumin. This report summarizes the results of a study of the effects of cortisone on antibody-antigen reactions and circulating antibody in the guinea pig. The observations indicate that administration of large doses of this hormone during the course of immunization of guinea pigs with egg albumin modifies the Arthus reaction and effects a significant reduction in circulating antibody. In confirmation of results of other investigators(3,4), pretreatment with cortisone fails to alter either active or passive anaphylactic shock.

Materials and methods. Cortisone acetate (Merck and Co.) was administered. Crystalline hen's egg albumin was prepared by sodium sulfate precipitation(5). A pooled rabbit anti-ovalbumin serum containing 0.77 mg of antibody nitrogen per ml was employed for passive sensitization. Guinea pigs weighing 200 to 300 g were injected intravenously with 0.5 ml of a 1:10 dilution of the serum, equivalent to 39 μg of antibody nitrogen. This amount of antibody was sufficient to cause anaphylactic death of 90% of control animals challenged intravenously 48 hours later with 1 mg of egg albumin. For active sensitization, animals were given 2 subcutaneous injections of 10 mg of egg albumin on alternate days. Fifteen days after the first injection, they were challenged intravenously

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TABLE I. Effect of Cortisone on Passive Anaphylaxis in Guinea Pigs.

Cortisone, mg/inj.	Total	Treatment before challenge, hr	No. of pigs	No. of deaths	No. of survivors
1	1	2	2	2	0
		4	3	2	1
		6	3	2	1
		8	2	2	0
		24	3	2	1
4	4	4	2	1	1
8	8	4	8	7	1
	8	8	7	7	0
	8	24	8	7	1
	24	21, 52, 69	2	2	0
	32	4, 21, 52, 69	8	8	0
Total cortisone-treated			48	42 (88%)	6
" controls			40	36 (90%)	4

with 1 mg of ovalbumin. In the study of the effect of cortisone on the Arthus reaction and on circulating antibody, a group of 60 male white guinea pigs weighing 500 to 600 g were inoculated subcutaneously with 0.5 ml of an emulsion prepared by blending 100 mg of heat-killed tubercle bacilli, 40 ml of "Falba," 1 g of egg albumin dissolved in 20 ml of saline, and 20 ml of paraffin oil(6). A second injection of 0.5 ml of the emulsion was administered 4 days later. Four, 10, 17, and 23 days after the second injection, the guinea pigs were challenged intracutaneously with 0.2 ml of an antigen solution containing 0.06 mg of egg albumin nitrogen. At the time of the last skin inoculation, a test for tuberculin hypersensitivity was performed with 0.1 ml of a 1:20 dilution of O.T. prepared by the Bureau of Animal Industry, U. S. Department of Agriculture. Three days later or 30 days following the first sensitizing injection, the animals were bled from the heart and killed with chloroform. Spleen, liver, and adrenals were weighed. Antibody nitrogen content of the sera was determined by the quantitative precipitin method of Heidelberger and Kendall and the micro-Kjeldahl technic(7).

Experimental results. A. *Effect of cortisone on passive anaphylactic shock.* Thirteen passively sensitized guinea pigs were treated with a single intramuscular injection of 1 mg of cortisone at 2, 4, 6, 8, or 24 hours before challenge. Thirty-three others were given injections of 8 mg of the hormone at intervals ranging from 4 to 69 hours prior to receiving the antigen. Since the interval between passive sensitization and challenge was 48 hours, those animals which received cortisone 69 hours before challenge were treated with cortisone prior to sensitization. Following the intravenous injection of antigen, all animals developed symptoms typical of anaphylactic shock; no significant differences were observed between the cortisone-treated group and the controls (Table I).

B. *Effect of cortisone on active anaphylaxis.* Fifteen guinea pigs undergoing active sensitization were treated daily with a single intramuscular injection of 1 mg of cortisone beginning on the first day of sensitization. Another 15 were given 2 mg of cortisone daily; 10 others received 5 mg of cortisone for 4 days prior to sensitization and for the next 10 days, after which time the dosage was reduced to 2.5 mg per day because of weight loss and deaths. Fifteen days after the first sensitizing injection of antigen, while still under treatment, the animals were challenged intravenously with 1 mg of egg albumin. As shown in Table II, treatment with cortisone neither inhibited sensitization nor modified the symptoms of anaphylactic shock, and deaths in all groups occurred within 6 minutes after receiving the antigen.

C. *Effect of cortisone on the active Arthus reaction,* on circulating antibody, and on the tuberculin skin test. Twenty of 60 guinea pigs sensitized with antigen incorporated in Freund's adjuvant mixture were treated daily from the onset of sensitization with 5 mg of cortisone for 18 days and then with 10 mg of the hormone for the next 12 days. The hor-

TABLE II. Effect of Cortisone on Active Anaphylaxis in Guinea Pigs.

Cortisone, mg/inj.	Total	No. of pigs	No. of deaths	No. of survivors
1	15	14	14	0
2	30	14	12	2
2.5–5	72.5	5	4	1
Total cortisone-treated		33	30	3
" controls		34	32	2

TABLE III. Effect of Cortisone on Active Arthus Reaction and Circulating Antibody in Guinea Pigs Sensitized with Crystalline Egg Albumin.

No. of pigs	Treatment	Skin reaction	Serum antibody conc. in μg of Ab N/ml, mean ± S.E.
17	Cortisone, 5 mg/day, 0-18th day; 10 mg/day, 18-30th day	Avg = .5 r* 3 hemorrhagic 9 red 5 pink	26 ± 7 3 of 16 with conc. >40
32	0	Avg = 4 h 18 hemorrhagic 12 red 2 pink	109 ± 17 21 of 28 with conc. >40

* Product of length, width and height measured in cm.

mone was administered in 2 divided doses given 12 hours apart. These dosages of 10 to 20 mg of hormone per kg of body weight, which are approximately 5 to 10 times greater than that generally employed in the treatment of man (100 mg per 50 kg), did not markedly affect the nutritional status of these larger guinea pigs. Most of the treated animals gained weight during the experimental period, although not as rapidly as did the 40 controls. Three of 20 treated animals and 8 of 40 controls died before the termination of the experiment.

Eight days after the first sensitizing injection of egg albumin in adjuvant, most of the animals produced a slight pink skin reaction to an intradermal test dose of antigen. With this first skin test, no differences were observed in the responses of the cortisone and control animals. Although the control animals responded with more intense Arthus reactions to succeeding skin tests, the reactions of the cortisone treated animals failed to increase in intensity. In Table III are presented the average skin responses to the last or fourth injection of egg albumin. Eighteen of 32 control animals or 56% produced a hemorrhagic Arthus reaction, whereas only 3 of 17 treated guinea pigs or 18% responded in a similar manner. By the χ^2 method, this difference could occur by chance alone in approximately once in 50 times. It is, therefore, probable that treatment with cortisone produced a significant inhibitory influence on the Arthus reaction.

The effect of cortisone on circulating antibody was striking. As shown in Table III, the mean serum antibody nitrogen concentration of the treated animals was less than one-fourth that of the controls (26 and 109 μg of antibody nitrogen per ml of serum, respectively). The probability of this result occurring by chance alone is approximately 1 in 1,000 as determined by "Student's" T test. A rough correlation similar to that previously observed in rabbits(2), between the serum antibody concentration and the severity of the skin response, was encountered. Only one of 3 treated animals and one of 15 controls which responded maximally to the antigen had less than 40 μg of circulating antibody nitrogen per ml of serum.

Since the guinea pigs in the present study were sensitized with antigen in Freund's adjuvant containing heat-killed tubercle bacilli, an opportunity for study of the effect of cortisone on the development of the tuberculin reaction arose. The average tuberculin reactions present 48 hours after an intradermal injection of 0.1 ml of a 1:20 dilution of "Old Tuberculin" are given in Table IV. It is apparent that treatment with cortisone diminished the tuberculin response. The skin le-

TABLE IV.
Effect of Cortisone on Tuberculin Skin Test.

No. of pigs	Treatment	Tuberculin reaction
16	Cortisone, 5 mg/day, 0-18th day; 10 mg/day, 18-30th day	Avg = .1 p* 3 red 13 pink
31	0	Avg = 1 r 13 red, necrotic center 11 red 7 pink

* Product of length, width and height measured in cm.

TABLE V. Effect of Cortisone on Weights of Spleen, Liver and Adrenals.

No. of pigs	Treatment	Wt of following organs in g, Mean ± S.E.		
		Spleen	Liver	Adrenals
17	Cortisone, 5 mg/day, 0-18th day; 10 mg/day, 18-30th day	.77±.05	27.7±1	.20±.01
26	0	1.22±.05	24.4±1.1	.27±.01

sions of the treated animals were smaller and less severe than those of the controls. Furthermore, none of the lesions of the treated animals became necrotic, whereas one-third of the control lesions showed necrotic centers.

The organ changes produced by cortisone (Table V) were similar to but less extensive than those which have been previously observed in the rabbit(8). The spleen and adrenals were distinctly smaller than those of the controls, weighing respectively two-thirds and three-fourths as much. The liver was normal in size and appearance and no lipemia was visible.

Summary. 1. Large doses of cortisone failed to protect guinea pigs against active or passive anaphylactic shock. These results are in agreement with those of other investigators(3,4). 2. Treatment with this hormone during period of active sensitization diminished the intensity of the Arthus reaction and the quantity of circulating antibody produced. Neither of these effects was as extensive as those observed in rabbits following the administration of comparatively smaller doses of cortisone(1,2). Similarly, the anatomic changes produced by cortisone were less striking in the guinea pig than in the rabbit (8). These findings suggest that the guinea pig is considerably more resistant to the action of cortisone. The failure of cortisone to abolish the active Arthus reaction and possibly its failure to alter active anaphylactic shock might have been related to the incomplete suppression of circulating antibody under the experimental conditions employed.

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A Phylogenetic Study of the Milk "Let-Down" Hormone. (19566)

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Mammals are divided into 3 divisions. The monotremata or egg-laying mammals are the most primitive derivatives from reptilian stock. Their mammary glands open on the skin in association with hairs but are without

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* Contribution from the Department of Dairy Husbandry, Missouri Agric. Exp. Sta., Journal Series No. 1307.

nipples. The eutherian mammals include all those in which the young are born alive (viviparous). This group includes both marsupial and placental mammals. The former group are without a true placenta and usually possess a pouch in which the offspring are kept after birth. Nipples are always present. The young become attached to a nipple for a considerable period during early pouch life. Only later do they become detached from the nipple during the interval between nursing periods. In placental mammals which are more mature when born, the young generally nurse only at intervals. (During the interval between nursing the young are not attached to the nipples.) In certain placental mammals, it has been shown that the milk secreted in the mammary glands only becomes available to the nursling or the milker upon the nervous stimulation of the nipples. This causes the discharge of the posterior pituitary milk let-down factor which is thought to cause the contraction of the myoepithelial cells surrounding the alveoli and ducts, so releasing the milk.

Extensive assays of the amount of milk "let-down" hormone in the pituitaries of dairy cattle have been made possible by the use of the lactating sow(1). In connection with these studies, it seemed of interest to determine if milk "let-down" hormone was secreted by all types of mammals. Such information would throw some light on the milk "let-down" mechanism of primitive mammals. The question of whether the mechanism for the release of the "let-down" hormone is the same in monotremes without nipples as in the more developed mammals is unanswered. This problem is also unsolved in the case of the marsupials whose young are attached continuously to the nipples for a period. The first point to be cleared up is whether or not these animals have a posterior pituitary milk "let-down" hormone. Further, it seemed of interest to determine the amount of milk "let-down" hormone in a series of placental experimental animals with the view of determining the species and sex variation. The object of this paper is to present the results of a phylogenetic study of mammalia in regard to the presence of the milk "let-down" hormone in the neuro-hypophysis of representative species, and if present to estimate the amounts present in widely differing forms.

Material and methods. As a representative of the monotremata, the pituitary of a single female echidna, the Australian ant-eater, was obtained from the Auckland (N.Z.) Zoo. The animal choked to death on a piece of ground meat. The pituitary was removed immediately the accident was discovered and placed in acetone. The animal was not lactating, nor did the ovary appear active at the time of death. Two representative marsupials were obtained. The Australian phalanger (*Trichosurus vulpecula*) has been introduced into New Zealand, where it is commonly called an opossum. These animals were trapped around Palmerston North, N.Z. The pituitaries of males, non-lactating and lactating females were dissected out and immediately dropped into acetone. This was done as soon as the animal was taken from the trap. Representative samples of each type of pituitary were obtained. A wallaroo in the Auckland Zoo was found dead in its paddock after appearing in perfect health the day before. Three of the 4 mammary glands were inactive but one teat was greatly elongated and the gland was much enlarged. The exact "pouch" age of the young was not known but since the non-used mammary glands were considerably smaller, some time must have elapsed for the involution to have proceeded so extensively. The time elapsing from its death until the pituitary was placed in acetone would undoubtedly decrease the amount of hormone. Of the placental mammals, posterior pituitary lobes were obtained from the following: Normal male and non-lactating female albino rats of the Wistar strain, normal male and non-lactating female rabbits, Romney Marsh ewes in mid-lactation, a large white sow at end of lactation, and dairy calves, heifers and lactating mature cows. The rat and rabbit pituitaries were obtained in Missouri. The other animals were Ruakura stock. For comparison a marlin (sword fish) pituitary was obtained. A period of about 24 hours elapsed between the time of capture and placing the pituitary into acetone. The resulting assay can only be qualitative due to the loss of potency caused by enzyme activity.

TABLE I. The Milk "Let-Down" Hormone in the Posterior Lobes of Representative Mammals.

Animal	Sex	No. tested	Avg wt of dried glands (mg)	Avg total activity (I.U.)	Specific activity (I.U./mg)	Body wt (lb)	Activity/lb body wt
			Posterior lobe				
Rabbit	♀	6	1	.22	.2	6.45	.03
	♂	3	1	.27	.3	8.71	.03
Rat	♀	22	.27	.22	.8	.35	.6
	♂	20	.25	.18	.7	.4	.4
			Whole pituitary				
Phalanger	♀	17	4.7	.21		5†	.04
	♀ L*	14	5.3	1.1		5†	.2
	♂	27	5	1.1		5†	.2
			Posterior lobe				
Walleroo	♀	1	60	2.7			
Echidna	♀	1		2.3			
			Whole pituitary				
Marlin	♂	1	24	.7		260	.003‡
			Posterior lobe				
Cows	L	9	63	31	.5	766	.04
Calves	♀	14	32	51	1.6	184	.3
	♂	1	116	20		82	.2
			Whole pituitary				
Sheep	♀ L	5	257	4.6		120†	.04
			Posterior lobe				
Pig	♀ L	1	16	13	.8	270	.05

* L = Lactating. † Approx. ‡ Gland removed 24 hr after death.

Except where otherwise indicated, within 30 minutes after the animals were killed the entire neurohypophysis was dissected out, dropped into cold acetone and stored at —12°C. Usually the next day in the case of cattle the median eminence, the stalk and the posterior lobe were separated, further defatted and dehydrated in changes of acetone, dried *in vacuo* and weighed. The anterior lobe of the ewes could not be separated from the posterior lobe. The rat and rabbit posterior lobes only were dropped into acetone and dehydrated.

The milk "let-down" hormone was extracted and assayed by methods previously described (Whittlestone) (2). All assays are expressed as the milk "let-down" equivalent of a standard oxytocic solution.

Discussion. A factor or factors which will stimulate the "let-down" of milk in the sow has been shown to be present in the posterior pituitary of all classes of mammals (Table I). The echidna, a representative of the most primitive type of mammal, the monotremes, showed a very definite response (2.3 I.U.) even though some time elapsed after its death before the pituitary was removed. Since in this form teats are not present, the nuzzling of the gland areas must be sufficient to cause discharge of the milk "let-down" hormone.

The pituitaries of two types of marsupials showed responses. The pituitary of a large lactating wallaroo, a type of kangaroo, showed the presence of only slightly more hormone than that of the echidna. It is suspected that this is due to enzymatic losses before the gland was placed into acetone. The phalanger pituitaries are of especial interest since they were collected quickly following death. It is notable that the lactating females contained five times more hormone than the non-lactating females. The high level of hormone in the male pituitary is interesting.

A large representative group of placental mammals has now been studied. In each of them the presence of the factor causing milk "let-down" has been demonstrated. The amount of "let-down" hormone in terms of body weight shows quite large variations between species. The adult rat shows about 10 times the relative activity found in the rabbit, sheep, pig, and mature cow.

The difference in relative activity between the glands of the calf and the mature cow is very great. The calf shows an amount of milk "let-down" hormone per unit of body weight which is almost 10 times as great as that shown by the older cows. Work in progress may throw light on the factors influencing the amount of hormone in the dairy cow (Whittlestone *et al.*)(1).

All assays of milk "let-down" hormone were carried out on the lactating sow. The duration of the activity was used as a basis for comparison. It is interesting to note that despite their widely different sources all preparations appeared to exert the same action on the sow mammary gland. The changes in the tone of the gland as detected by the milker appeared to be the same regardless of the origin of the hormone. Similarly the rate at which the milk was ejected was not influenced by the source of the milk "let-down" substance. There is no reason to suspect that the active principle or principles from the posterior pituitary gland is different even in such widely separated creatures as the pig and the echidna.

It is worth noting that the pituitary gland of the fetus of one of the cows studied in another project, to be reported later, was examined for milk "let-down" activity and found to be inactive. This fetus, which was 12 weeks old, had not reached a stage of development at which milk "let-down" hormone was present. The dry weight of the whole posterior pituitary gland was 90 mg.

In addition to the mammalian pituitaries, the presence of a milk "let-down" factor was also demonstrated in the pituitary of a 260 lb marlin or sword fish. While this might appear surprising, it should be pointed out that both the oxytocic and vasopressor (antidiuretic) hormones cause the milk "let-down" phenomena (Whittlestone, 1952)(2). It is possible that the "let-down" effect observed might be due to the antidiuretic factor.

These data on the widespread presence of the factor influencing milk "let-down" may be compared with the assays of mammalian pituitaries for the presence of oxytocic and vasopressor factors by Geiling(3), who has shown that both hormones occur in whales, porpoise, armadillo, and even in chickens.

Summary. 1. A factor or factors which will stimulate the "let-down" of milk in the sow has been shown to be present in the posterior pituitary gland of representatives of the 3 divisions of mammals, the monotremes, the marsupials, and the placentals. 2. The pituitary of a marlin or sword fish also caused milk "let-down" in a sow.

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Effect of Acetylcholine on Mammary Gland of the Lactating Sow. (19567)

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The role of the neurohypophysis as the source of the hormone controlling milk let-down has been clearly established by many workers since the first observations of Ott and Scott(1) on the effect of pituitary extracts on the mammary gland. Cross and Harris(2) have recently shown that the electrical stimulation of the pituitary stalk of the rabbit will result in the let-down of milk. There is evidence that both of the posterior pituitary hormones will cause milk let-down(3). Following milk let-down, it has been shown by Cross(4), and Peeters and Coussens(5) that antidiuresis occurs. Despite the clearly hormonal aspects of the control of milk let-down,

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* Contribution from the Department of Dairy Husbandry, Missouri Agric. Exp. Sta., Journal Series No. 1308.

TABLE I. Effect of Acetylcholine on Milk Let-Down.

Exp.	Treatment	Time from inj. to response, sec	Duration of milk let-down, sec	Milk yield, g	Remarks
I	.5 I.U. oxytocin	13.4	35.9	51.8	Sow in milk 1 wk.
	.2 g acetylcholine	13.9	40.3	12.7	Low milk yield.
	.5 I.U. oxytocin	13.9	30.2	61.4	
II	.2 g acetylcholine	—	—	—	No response.
	.5 I.U. oxytocin	15.9	47.1	172.9	Sow in milk 3 wk.
	.3 g acetylcholine	—	—	—	No response.
III	.5 I.U. oxytocin	15.7	45.8	71.8	Sow in milk 2 wk.
	.2 g acetylcholine	16.5	23.9	3.1	Low milk yield.
IV	.5 I.U. oxytocin	19.2	29.6	53.9	Sow in milk 2 wk.
	.2 g acetylcholine	16.9	21.3	48.4	
	.1 g ” .5 g prostigmine	15.2	19.3	52.6	
V	.5 I.U. oxytocin	15.4	49.6	68.3	Sow in milk 8 days.
	.2 mg Carbachol	15.6	42.6	12.4	Low in milk yield, sow sat down.
	.1 mg ”	27	15	7.7	Abnormal delay after injection.

the possibility of an alternative neural pathway exists. That adrenalin is not the normal mediator is indicated by the research of Ely and Petersen(6), and Whittlestone(7) who showed the inhibiting role of this substance on the mammary gland. It is possible that the fibers supplying the posterior lobe are adrenergic. However, this action would be completely masked by the inhibiting action of adrenalin on the milk let-down process when this is used as a criterion of activity. Pickford (8) has shown that acetylcholine will evoke antidiuresis and the concentration of chloride in the urine of the dog. This suggests the possibility of the posterior pituitary being innervated by cholinergic fibers. Petersen(9) showed that acetylcholine will permit the removal of milk from the secreting tissue of a perfused mammary gland. This observation was complicated by the fact that no response was obtained from carbaminoylcholine.

In the light of our experience with the lactating sow as an experimental animal for the study of milk let-down(3) and in view of the results obtained by the injection of oxytocin and adrenalin in different orders(7), it was thought worthwhile to study the action of acetylcholine and its derivatives on the mammary gland of the intact animal. The present paper describes the results obtained from such preliminary experiments.

Material and methods. As in previous milk let-down experiments the standard of response has been taken as that evoked by 0.5 I.U. of oxytocin. A lactating sow was brought to a specially designed milking machine(10). Injection of the substance to be studied was made via an ear vein. The interval between injection and let-down and the duration of the latter as judged by the flow of milk obtained by hand-milking one of the front teats were recorded. The effects of a series of injections of 0.5 I.U. of oxytocin on the milk let-down time of the sow have been studied(3). Normally the first dose elicits a response of much greater duration than the second and subsequent injections and a slow decrease in duration of let-down follows with the third and subsequent injections. A minimum period of 5 minutes was allowed between injections in order to minimize interaction between consecutive doses. The amount of milk drawn by machine from the teats not hand milked was also recorded.

Experimental. In the first experiment it was observed that the injection of 0.2 g of acetylcholine into a lactating sow caused a milk let-down period of 40.3 seconds duration (Table I). However, the small amount of milk obtained compared with that obtained following the injection of oxytocin suggests the action of a substance with properties different from the latter. Since the time from injection to response is the same as that following the injection of oxytocin it is obvious that the immediate action of acetylcholine was directly upon the mammary gland rather than on the pituitary. That it also stimulated the pituitary, however, is quite possible.

In a second sow neither 0.2 g nor 0.3 g of acetylcholine was effective. A third sow was stimulated to let down her milk but only a small amount of milk was obtained by the machine. A fourth sow was stimulated by 0.2 g of acetylcholine to give a normal let-down time and a normal amount of milk. When 0.1 g was injected together with 0.5 g of prostigmine there followed the expected augmentation of the action. This result was typical of a number of experiments which indicated the anticholine esterase action of prostigmine.

The derivative of choline, carbaminoylcholine (carbachol) at 0.2 mg and 0.1 mg levels gave graded responses. The milk yield may have been reduced due to the fact that the sow sat down during the milking process.

Discussion. These experiments clearly indicate that acetylcholine will cause milk let-down in the sow. From the fact that the time interval from injection of acetylcholine to milk let-down is essentially the same as that observed after oxytocin injection, it was concluded that it acted directly upon the mammary gland. Further research will be required to determine whether acetylcholine also stimulates the pituitary to release the let-down hormone which Pickford's results would indicate.

Two points of difference in the action of acetylcholine as compared to oxytocin were observed. In some animals the amount of milk removed was low in comparison to the duration of let-down time. The second difference was the variability in the response of some sows.

Prostigmine, which inhibits the enzyme choline-esterase, had the expected augmenting activity upon acetylcholine. The fact that small amounts of the closely related derivative carbaminoylcholine produced a let-down effect in the intact sow but not in the isolated udder of the cow(9) cannot be explained.

Summary. Acetylcholine was shown to cause the let-down of milk in the intact lactating sow. The time interval from injection to milk let-down was believed to indicate direct action upon the mammary gland, although action on the pituitary may also occur. The let-down effect was augmented by the anticholine-esterase action of prostigmine. Small amounts of carbaminoylcholine, a derivative, had let-down action similar to acetylcholine.

The writers wish to thank Messrs. D. M. Smith and E. D. O'Reilly of the Ruakura Piggery for their assistance in making available the assay animals and for the intravenous injection of the preparations.

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Source of Secretion of Milk "Let-Down"* Hormone in Domestic Mammals.

(19568)

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Early anatomists, failing to observe secretory cells in the neurohypophysis, were not inclined to consider this organ an endocrine gland. When physiologists showed that extracts of the posterior lobe possessed a high degree of pharmacological activity on the uterus, blood vascular system, kidneys and mammary gland, workers were stimulated to determine the origin of the active principles. Herring(1) suggested that the cells of the pars intermedia invaded the posterior lobe and became transformed into hyaline bodies (Herring-bodies) which represented the secretion. That Cushing(2) still held this view up to 1933 is implied by the following quotation: "The active principles of the posterior lobe and its several fractions must under all circumstances primarily be derived, not from the pars nervosa, but from its epithelial investment, the pars intermedia." In 1935 Geiling and associates(3) commenced the publication of data which proved that in a number of species of animals, although the pars intermedia was lacking, the vasopressor (antidiuretic) and oxytocic hormones were present in posterior lobe extracts. Recently Bargmann and Scharrer(4) suggested that the hormones of the posterior lobe were secreted by neurosecretory cells of the hypothalamus and were transported down the axons to the pituitary stalk and pars nervosa.

Using the lactating sow as an assay animal, Whittlestone(5) has shown that highly active vasopressin as well as oxytocin will cause milk let-down. It seemed of interest therefore to determine whether the theory of hypothalamic secretion applied to the milk let-down factors of the posterior lobe.

Experimental. A number of calves, heifers and lactating cattle, a few ewes and one sow, were slaughtered during the course of other experiments at Ruakura. Within 30 minutes the entire neurohypophysis was dissected out and dropped into cold acetone. Later the median eminence, the stalk and the posterior lobe were separated, further defatted and dehydrated in changes of acetone, dried *in vacuo* and weighed. The dried material was ground up with sand, extracted with a known volume of 0.05% acetic acid, centrifuged and the supernatant liquid heated in a water bath. The resultant precipitate was centrifuged off and the solution stored in a refrigerator until assayed. The extract of the median eminence was sometimes difficult to clarify but the cloudy extract was found to produce no ill effects in the assay animals. The activity of the extracts was estimated by injection into the lactating sow and determining by hand milking the duration of action of the "let-down" hormone(5). Comparisons were made with a standard solution of oxytocic hormone prepared from lyophilized Pitocin kindly supplied by Parke, Davis & Co., Detroit. (This preparation is checked occasionally for loss of potency against an International Standard powder.) In this series of assays, the sows were first injected with 0.5 I.U. of solution and the time during which milk could be removed was noted. This was followed by an injection of the extract to be assayed. The response of the latter was then matched by a further injection of the standard. Milk let-down may be detected in the sow following

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* Recently Cowie, et al., (1951) suggested that the term milk "ejection" be used in preference to milk "let-down" to indicate the effect of alveolar and ductal contraction of the mammary gland. Since the term milk "ejection" connotes the forceful passage of milk to the exterior, which does not necessarily occur, the term milk "let-down" as a prelude to the act of milk removal seems more appropriate. (Cowie, A. T., Folley, S. J., Cross, B. A., Harris, G. W., Jacobsohn, D., and Richardson, K. C., *Nature*, 1951, v168, 421.)

† Contribution from the Department of Dairy Husbandry, Missouri Agric. Exp. Sta., Journal Series No. 1310.

TABLE I. The Distribution of Milk Let-Down Activity in the Mammalian Neurohypophysis.

	No.	Age	Body wt, lb	Median eminence			Stalk			Posterior lobe		
				Wt (mg)	Total activity (I.U.)	Specific activity (I.U./mg)	Wt (mg)	Total activity (I.U.)	Specific activity (I.U./mg)	Wt (mg)	Total activity (I.U.)	Specific activity (I.U./mg)
Calves		Wk										
	102	4	81	523	—	—	19	.7	.035	129*	14	—
	104†	4	82	614	—	—	10	.6	.06	116*	20	—
	12	4	93	378	.1		9	.5	.06	20	22	1.1
	105	8	113	121	—	—	9	.23	.025	Lost		
	106	8	123	132	.15		10	.1	.01	31	31	1
	11	8	132	477	Trace		8	.7	.09	25	28	1.1
	32	12	190	230	—	—	10	.1	.01	31	59	1.9
	30	12	148	60	—	—	2‡	1	.5	22	55	2.5
	10	12	187	246	—	—	9	Lost		26‖	16	.6
	107	16	207	217	—	—				32	74	2.3
	101	16	182	101	Trace		4	.6	.15	26	52	2
	1	18	156	152	Trace		3	.6	.2	26	23	.9
	2	18	205	292	—	—	10	.6	.06	49	49	1
	3	18	175	212	—	—	13	1.3	.1	25	28	1.1
	18	18	127	299	—	—	7	.7	.1	29	75	2.6
	100	20	251	484	—	—	12	—	—	60	108	1.8
	33	20	301	179	Trace		11	.55	.05	33	58	1.75
	26	20	291	168	—	—		Lost		32	51	1.6
	17§	64	560	354	—	—	16	.8	.05	71	178	2.5
	18	67	460	262	.5		13	.9	.07	44	79	1.8
Cows		Yr										
	608	2	662	264	—	—	21	.3	.01	57	37	.6
	73	3	580	330	—	—	12	.6	.05	48	23	.5
	89	3	597	322	—	—	24	.6	.025	59	22	.4
	598	3	763	267	.3	.001	21	.8	.04	82	31	.4
	563	5	708	341	—	—	25	.2	.01	23	13	.6
	T5	5	797	279	—	—	21	.08	.004	79	40	.5
	T6	5	756	311	Trace		20	.08	.004	83	58	.7
	45W	5	1009	328	—	—	18	1	.055	41	21	.5
	48W	5	1026	306	—	—	8	.6	.07	95	38	.4
Sheep		Yr								Whole pituitary		
	P147	5		125	.15		9	.18	.02	423	9	
	C372	”		233	—	—	5	.05	.009	139	5.5	
	P 13	”		146	—	—	14	1.4	.1	235	1.8	
	C384	”		261	—	—	5	.15	.03	189	1.7	
	P 45	”		389	—	—	Lost			297	4.8	
										Posterior lobe		
Pig				262	—	—	2	—	—	16	13	.8

* Whole pituitary gland. † Male calf. ‡ Incomplete stalk. § Pregnant heifer, No. 18 non-pregnant identical twin. ‖ Posterior lobe not completely separated.

injections of as little as 0.02-0.03 unit of oxytocin.

Results. In the dissection of the neurohypophysis there are no clearly defined boundaries of the median eminence of the hypothalamus. In the present study the area immediately superior to the attachment of the stalk surrounding the third ventricle was included as the "median eminence." Since its boundaries were poorly defined, a considerable amount of adjacent tissue was included.

It was noted in the dissections that the fibers of the superior end of the stalk are attached to the tissue surrounding the third ventricle. In most of the dissections the line of demarcation was arbitrarily set by cutting the superior end of the stalk close to the median eminence. Under these conditions the fibers of the stalk attached to the surface of the third ventricle would be included as part of the median eminence. In a few cases the fibers of the stalk were dissected free of their attachment to the surface of the third ventricle.

It will be observed that the median eminences of most cattle contain little or no milk "let-down" hormone (Table I). In a few cases there were traces to measurable amounts. Such activity is probably due to stalk fibers retained by the median eminence after dissection.

These data indicate that the suggestion of Bargmann and Scharrer regarding the origin of the posterior pituitary hormones in the hypothalamus apparently does not hold for the milk "let-down" hormone. If the hormone is secreted in that division of the neurohypophysis it must be in an inactive form, becoming active in its passage down the stalk.

In this connection it is interesting to note that the stalk in every case contained measurable amounts of hormone. However, the amount of hormone in the stalk per mg of acetone-dried tissue (specific activity) was roughly of an order of one-tenth or less that of the posterior lobe. These observations suggest that the movement of hormone is from the posterior lobe to the stalk rather than from the median eminence to the stalk.

Summary and conclusions. 1. The neurohypophyses of a series of animals were separated into the median eminence, stalk and posterior lobe. The acetone-dried tissue in each division was extracted and assayed for milk "let-down" hormone content, using lactating sows as assay animals. 2. The median eminence usually contained an insufficient amount of hormone to cause milk "let-down" in the sow. In a few cases some activity was observed. 3. It was concluded that the milk "let-down" hormone is either not secreted in the hypothalamus or, if secreted there, exists in an inactive form which becomes active only as it passes down the stalk into the posterior lobe. Since the posterior lobe contained roughly 10 times the amount of hormone present in the stalk per unit of dry matter, it seems more probable that the posterior lobe is the source of the milk "let-down" hormone.

The writers wish to indicate their gratitude to Mr. D. M. Smith for permission to use the facilities of the Piggery and to Mr. E. D. O'Reilly for his handling of the pigs and for his carrying out the many injections involved.

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Effect of Epinephrine and Insulin on Tissue Electrolytes in Normal and Demedullated Rats. (19569)

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A reduction in the blood potassium level has been reported following the administration of insulin and epinephrine in normal animals and man(1-4). Considerable evidence in the literature(5,8), however, indicates that either epinephrine or insulin reflexly releases the other humoral agent apparently through the mediation of altered glycemic levels. In view of the interaction between these two endocrine substances it appeared desirable to investigate further the effect of insulin and epinephrine on the ionic content of plasma and selected tissues.

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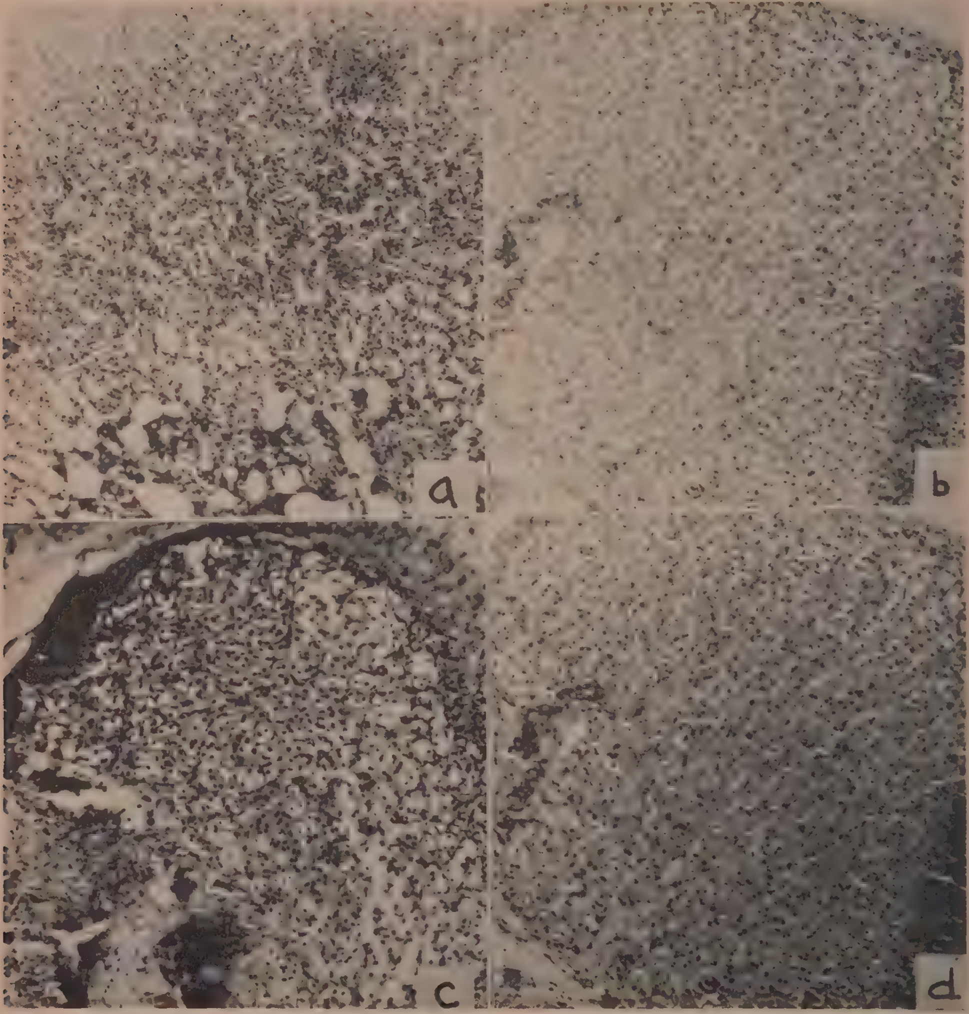

FIG. 1. Sections of adrenal glands. (a) Normal sham-operated. (b) Demedullated 21 days; non-treated. (c) Demedullated 21 days; epinephrine-treated. (d) Demedullated 21 days; insulin-treated. Note that the regenerated adrenal cortices appear histologically normal. (All sections × 80.)

Materials and methods. The experiments were designed to compare the effects of epinephrine and insulin on tissue electrolytes and water content in intact groups of rats and rats with apparently functional adrenal cortices but no medullary tissue (adrenal medulla). Greep and Deane(9) showed that approximately 21 days after adrenal-enucleation the regenerated adrenal cortices have attained normal functional capacity. Accordingly, in the experiments reported here groups of sham-operated and demedullated male Wistar rats were identically prepared for use in the experiments 21 days after surgery, as follows: Adrenal-enucleation was done by the method of Ingle and Higgins(10). The sham operation was done similarly except for the final stage of enucleation. After the surgery the rats were maintained on chow biscuit and a 1% NaCl drinking solution *ad libitum* for the next 10 days. The salt solution was shown(9) to be necessary for the adrenal-enucleated during the first 8-10 days of adrenocortical regeneration. On the 11th day after surgery the 1% NaCl solution was replaced by tap water for the next 11 days. At the end of the 21-day post surgical period the sham-operated and demedullated were fasted overnight and treated as one of the following groups: No treatment (controls); epinephrine injected; and insulin injected. The rats were lightly anesthetized with n-methylcyclo-hexenyl-methyl barbituric acid (EVIPAL) before epinephrine or insulin was administered, and before tissues were taken for analyses. Epinephrine (Adrenalin tablets; Parke, Davis & Co.) was prepared fresh in physiological saline and administered subcutaneously at a dose level of 0.04 mg/100 g body weight (approx. 0.5 ml). Insulin (ILETIN, regular; Lilly) was administered subcutaneously at a dose level of 0.5 *U*/rat (0.6 ml). Cardiac blood was obtained from the control rats immediately after they were anesthetized without further treatment, and at the end of 60 minutes from the rats injected with epinephrine or insulin. Specimens of gastrocnemius muscle and liver were taken immediately after the blood was obtained. The procedures followed for the analyses of electrolytes and water composition of plasma, skeletal muscle, and liver were the same for all animals and have been described in detail in a previous report(11). Tissue chloride content was determined by the method of Van Slyke(12); and plasma chloride by the method of Schales and Schales(13). Plasma glucose was determined by the photometric method of Kingsley and Reinhold(14). Tissue and plasma sodium and potassium were determined with the aid of an internal lithium standard flame photometer.

Results. Photomicrographs of representative histological sections of adrenal glands from normal sham-operated and demedullated rats are presented in Fig. 1. It is apparent that except for the absence of medullary tissue in the glands of the latter group that the adrenal cortices appear histologically normal with good zonal regeneration.

The results of analyses of plasma, skeletal

TABLE I. Comparison of Tissue Water and Ionic Content 60 Min after Epinephrine and Insulin in Normal and Adrenal-Demedullated Rats.

Tissue constituent		Normal sham-operated			Adrenal-demedullated		
		Controls (6)	Epinephrine* (5)	Insulin† (6)	Controls (8)	Epinephrine (7)	Insulin (8)
Plasma‡	% H_2O	93.2 ± .14§	93 ± .11	92.1 ± .28**	93 ± .09	92.5 ± .07	92.7 ± .1
	Cl mEq.	112 ± 1.1	104.9 ± 1 **	109.1 ± 1.2	110 ± .8	106.9 ± 1.1 ¶	110.3 ± .5
	K "	4.97 ± .21	4.29 ± .09**	3.94 ± .24**	5.58 ± .27	4.38 ± .19**	4.86 ± .2
	Na "	154.3 ± 1.1	151.1 ± 2	161.5 ± 1 **	153.3 ± .6	155.3 ± .9	162.4 ± 1.1**
	Glu. mg %	134 ± 7	294 ± 11	34 ± 4	116 ± 6	283 ± 12	26 ± 3
Muscle‖	H_2O g	757 ± 1	761 ± 1.5¶	757 ± .7	757 ± 1.1	763 ± .8**	756 ± .8
	Cl mEq.	10.3 ± .3	12.6 ± .3**	9.5 ± .2	10.6 ± .2	12.7 ± .4**	8.9 ± .2**
	K "	97.8 ± 2.3	88.6 ± 1.2**	98.4 ± 4.3	93.7 ± .9	90.5 ± .3**	96.8 ± 2.2
	Na "	31.5 ± 2.7	40.3 ± 1 ¶	28.6 ± 3	33.7 ± .8	38.2 ± 1 **	31.3 ± .9
Liver	H_2O g	698 ± 1.4	694 ± 3.1	692 ± 2.1¶	696 ± 3.2	694 ± 1.8	692 ± 1.1
	K mEq.	80.6 ± 3.3	78.4 ± 3.5	82.9 ± 3.4	78.7 ± 1.5	85.9 ± 2.3¶	86.5 ± 2.1**
	Na "	35.5 ± 2.4	40.9 ± 2.9	34.9 ± 1.7	36.5 ± 1.7	32.5 ± 1.8	33.8 ± 1

* Epinephrine sub-q at dose level of .04 mg/100 g body wt. † Insulin sub-q at dose level of .5 *U*/rat. ‡ Electrolyte conc. expressed as mEq./kg plasma water. § Mean ± S.E. ‖ Muscle and liver content expressed per kg wet tissue. Differences between means which were significant are indicated: ¶ P values = .05–.01; ** P values = <.01. No. of rats in each group in parentheses.

muscle and liver of the sham-operated and demedullated groups of rats are presented in Table I. The respective mean values of the tissue composition determined in the epinephrine and insulin treated groups were compared with those of the controls to determine whether the difference between the means were statistically significant. Inspection of these data show the following significant changes: *After epinephrine treatment*—Plasma chloride and potassium concentration were significantly decreased. Muscle water, chloride and sodium content were significantly increased; but muscle potassium was decreased. These changes were found in both the *sham-operated and the demedullated* groups. An increase in liver potassium was determined in the demedullated group alone. *After insulin treatment*—Significant changes in the *sham-operated* group were: decreased plasma potassium and increased plasma sodium concentration; and decreased plasma and liver water content. Significant changes in the *demedullated* group were: increased plasma sodium concentration; decreased muscle chloride and increased liver potassium content. In summary, the results showed that in both the sham-operated and demedullated groups there was a consistent pattern of altered electrolyte composition in certain tissues 60 minutes after epinephrine injection. The effect of insulin on tissue ionic composition was not consistent in the sham and the demedullated groups except for the significantly increased plasma sodium concentration. Particularly relevant to the present study was the evidence that epinephrine induced a significant reduction in the plasma potassium concentration in the sham and the demedullated groups whereas following insulin administration the plasma potassium concentration was decreased in the sham but not in the demedullated group.

Discussion. The alterations in water and ionic content of tissues reported here after epinephrine administration in sham-operated and demedullated groups of rats were the same (except for the increased liver potassium in the demedullated group) as reported(11) in intact and adrenalectomized rats 60 minutes after epinephrine administration. These data indicate that epinephrine's effect on certain tissue ionic constituents was consistently the same pattern in several different kinds of animal preparation. An explanation of the changes in tissue water and ionic content after epinephrine was presented in the discussion of a previous report(11).

It has been shown(5-8) that certain phases of carbohydrate metabolism and indices referable to increased anterior pituitary-adrenocortical activity believed to be an effect of insulin, *per se,* were actually responsible to an epinephrine action. Apparently the insulin-induced hypoglycemia "reflexly" elicited increased secretion of epinephrine. Since the evidence presented here and in related studies (15,16) indicates that epinephrine has a significant role in the physiological regulation of potassium balance in the fluid compartments of the body, then the difference in insulin's effect on the plasma potassium concentration in the sham and demedullated groups can be explained as another illustration of the interaction of these 2 humoral agents in the intact animal.

Summary. 1. The effect of epinephrine and insulin administration on the water and ionic content of plasma, skeletal muscle, and liver was compared in normal sham-operated and adrenal demedullated (21 days after surgery) groups of rats. Histological evidence was presented that the regenerated adrenal cortices appeared normal and medullary tissue was absent. 2. A significant reduction in plasma and muscle potassium concentration was determined 60 minutes after epinephrine administration in the sham and demedullated groups. A decreased plasma potassium level 60 minutes after insulin administration was found in the sham group but not in the demedullated group. However, a significant increase in the plasma sodium concentration after insulin was determined in both sham and demedullated groups. 3. The results were discussed as indicating that the apparent effect of insulin upon the plasma potassium level in the intact animal was the resultant of "reflexly" elicited epinephrine secretion following insulin-induced hypoglycemia and not the effect of insulin *per se.*

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The Effect of Diet on Riboflavin and Xanthine Oxidase in Rat Liver and Intestine.* (19570)

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The effect of low protein and low riboflavin diets on liver riboflavin has been reported previously(1,2). The present study shows a proportional reduction in the riboflavin content of the sedimentable particles and the supernatant fractions of a liver homogenate in both of these types of dietary restrictions. The corresponding changes in intestinal riboflavin, and the independent fluctuation of total riboflavin vs. the flavin enzyme, xanthine oxidase, in both liver and intestine have also been determined.

Methods. Male weanling rats were placed on 4 diets that varied as follows:

Diet	A	B	C	D
GBI Casein (vit. test)	31%	8%	31%	28.5%
Riboflavin (mg/100 g)	.5	.5	0	.5
Soy flour (50% protein)†	0	0	0	5

† A. E. Staley Mfg. Co., Decatur, Ill. Extracted soya flour.

The remainder of each diet consisted (in g/100 g) of: Crisco 4, Wesson oil 2, cod liver oil 1, Phillips and Hart salt mixture 4, and glucose (89—casein and soy flour). The vitamin supplement added (mg/100 g); nicotinic acid 2.5, calcium pantothenate 1.0, thiamine 0.4, pyridoxine 0.4, biotin 0.05, inositol 50.0, p-aminobenzoic acid 10.0, vit. B_{12} 0.05, mixed tocopherols 50.0, ascorbic acid 25.0, 2-methyl-1,4-naphthoquinone 0.25, choline chloride 100.0. In comparison with the control diet A, diet B was low in protein, C was low in riboflavin, and D contained soy flour as a source of the unidentified xanthine oxidase (X.O.) factor(3). After 2 or 4 weeks of *ad libitum* feeding, groups of rats from each diet were sacrificed by decapitation for analysis of the livers and small intestines. Riboflavin was determined microbiologically, using *L. casei* as the test organism and a Difco synthetic medium. The tissues were homogenized with cold 0.04 M phosphate buffer, pH 7.4, and aliquots were prepared and assayed by the procedure of Strong(4). The liver homogenates obtained at 2 weeks were also separated into a combined particulate (nuclei plus mitochondria plus microsomes) and supernatant fractions by centrifuging at 0-5° and approximately 30,000 x g for 90 minutes(5). The precipitate was washed by resuspending it in buffer and centrifuging again for 90 minutes; the wash was added to the super-

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* This work was supported by a grant from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

TABLE I. Liver and Intestinal Riboflavin and Xanthine Oxidase Values Obtained When Weanling Rats Were Fed the Indicated Diets for 2 to 4 Weeks.

		Diet			
		A	B	C	D
		Body weights (avg at 28 days)			
	Tissue analyzed	176	88	91	174
Riboflavin (μg/g wet wt)	Liver: Whole homogenate	29.8 ±2.6	17.3 ±1.1	12.4 ± .8	26.9 ±1
	Precipitate	16.2 ± .7	10.7 ± .8	8.3 ± .7	16.1 ±1
	Supernatant	9.6 ±1.5 (3)	5.9 ± .5 (6)	3.6 ± .3 (5)	8.6 ± .9 (4)
	Intestine	5.15± .21 (5)	4.30± .24 (8)	2.35± .12 (7)	4.93± .27 (9)
Xanthine oxidase* (mm³ O_2/283 mg/ 20 min)	Liver	25 ±3 (6)	.6 ± .6 (8)	19 ±2.4 (9)	32 ±2.4 (7)
	Intestine	10 ±1.5 (4)	1.5 ± .8 (8)	6.4 ±1.3 (9)	27 ±2.8 (6)

Values given are mean ± stand. error for the number of rats indicated in parentheses. Values for 8 weanling rats at start of experiment were: Body wt 59 g, liver riboflavin 24.2, intestinal riboflavin 4.65, liver xanthine oxidase 15.

* Values for xanthine oxidase are given as net oxygen consumption with hypoxanthine substrate per 20 min per Warburg flask containing 283 mg fresh tissue.

natant. Both the supernatant and precipitate fractions were assayed for riboflavin. An attempt was made to fractionate the livers obtained at 4 weeks in a sucrose medium(2). Insofar as could be determined the results were comparable with those obtained in phosphate buffer, but the large amount of sucrose created difficulties in some of the riboflavin assays. The liver and intestinal homogenates obtained at 4 weeks were also analyzed for xanthine oxidase(6).

Results. The results are recorded in Table I. The decreased values for liver riboflavin on a low protein or low riboflavin diet agreed with and confirmed previous reports(1,2). Intestinal riboflavin showed a similar response to these dietary deficiencies, but a low protein diet had less effect on intestinal than on liver riboflavin. Addition of soy flour as a source of the X.O. factor had no measurable effect on liver or intestinal riboflavin, though it did affect the xanthine oxidase content of these tissues. Riboflavin was lost proportionately from both the particulate and supernatant fractions as a result of feeding either a low protein or low riboflavin diet. This is in agreement with previous studies(1,2). Riboflavin was not lost selectively from the supernatant fraction, as occurred with the flavin enzyme, xanthine oxidase(5).

Liver and intestinal xanthine oxidase levels again(6) showed: 1) a marked loss of this enzyme from both the liver and intestine on a purified low protein diet, 2) a marked loss of this enzyme from the intestine when the diet contained adequate protein and riboflavin but inadequate X.O. factor, and 3) a marked influence of the X.O. factor on the intestinal xanthine oxidase and a much smaller effect on the liver enzyme. Liver xanthine oxidase was not depleted extensively by a riboflavin deficient diet (diet C vs. A). Previous studies indicating a more marked effect of riboflavin deficiency(7) were carried out under more prolonged dietary restrictions, and the results could have been influenced by other factors, such as inanition(6) and a prolonged deficiency of the X.O. factor. In the present studies, where an acute riboflavin deficiency was evidenced by growth failure and low liver riboflavin values, the liver xanthine oxidase was decreased, but the effect was not marked. The effect of diet C in depleting intestinal xanthine oxidase could be attributed to the absence of the X.O. factor, and the effect of a riboflavin deficiency *per se* could not be determined.

There was no correlation between the effect of diet on liver or intestinal total riboflavin and the xanthine oxidase activity of the tissue. This was especially clear in a comparison of intestinal values on diets A and D; total riboflavin was essentially the same, while xanthine oxidase on diet D was almost 3 times as high as on diet A. Similarly a comparison of the liver supernatant fractions, which contained all the xanthine oxidase(5), showed approximately a 3-fold difference between diets C and A in riboflavin content but only a 30% difference in xanthine oxidase activity. Hence, xanthine oxidase did not constitute a major fraction of the total riboflavin present in either liver or intestine, nor was it affected by diet in a manner characteristic of other flavin constituents.

Summary. 1. Weanling rats fed diets deficient in protein or riboflavin for 2 to 4 weeks had liver riboflavin levels that were reduced approximately 40% and 55% respectively; the corresponding reduction in intestinal riboflavin was approximately 15% and 55%. About 60% of the riboflavin in normal liver was in the sedimentable particles (nuclei plus mitochondria plus microsomes), and the above loss of riboflavin was proportionately the same from both the particulate and supernatant fractions. 2. The incorporation of soy flour in the diet as a source of the unidentified xanthine oxidase factor had no effect on the total riboflavin content of the liver or intestine, but did increase the liver and intestinal xanthine oxidases 30% and 170% respectively. The amount of riboflavin bound in xanthine oxidase represents only a very small fraction of the total riboflavin present. Liver xanthine oxidase was decreased only 25% by a riboflavin deficient diet.

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Growth of Newcastle Disease Virus in the Embryonated Egg. (19571)

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There have been many approaches to the study of the factors involved in the multiplication of viruses. The early investigators were obliged to study virus propagation in terms of the reaction that followed the introduction of the agent into animals, isolated organs, or tissues. The growing interest in the phenomenon of virus propagation has given rise to many new avenues of approach and numerous ingenious technics. Although a large body of data have been collected, it appears that much basic information regarding the activity of the virus particle is still lacking. Especially is this true in regard to the mechanism of the development of the infectious and the hemagglutinating properties of some viruses. While considerable data regarding the influenza virus have been accumulated(1-5) the literature revealed but limited information on the growth and behavior of the Newcastle disease virus. However, some of its physical, immunological, and infectious properties have been described (6-10).

Since Burnet has reported a similarity between the viruses of Newcastle disease and influenza(11) this study was undertaken to apply to the Newcastle disease virus some concepts of host-virus interplay that had been described for influenza. This was accomplished by investigating the production of the infective and hemagglutinative properties of Newcastle disease virus.

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Materials and procedures. Newcastle disease virus. The CRE strain of Newcastle disease virus, isolated from a fatal case of Newcastle disease in a chicken, was obtained through Dr. William L. Ingalls, Department of Veterinary Pathology, Ohio State University. The virus had been subjected to many passages through embryonated eggs. The dilutions of virus used for inoculating fertile eggs were made in sterile brain heart infusion broth (Difco, pH 7.4). *Irradiated Virus.* The procedure followed for the preparation of the irradiated viruses was that described by Henle and Henle(12) for the preparation of ultraviolet inactivated influenza virus. *Eggs.* The Wayne strain of Leghorn eggs incubated at 37°C was used throughout this study. The age of the embryos used will be indicated in the experimental section to follow. *Inoculation procedure.* After candling each egg for viability, a small portion of the egg shell over the embryo was removed. The shell membrane was then pierced with a tuberculin syringe fitted with a No. 27 gauge needle. The inoculation was made into the allantoic sac. Sterile paraffin was used to seal the site of injection. *Harvesting procedure.* To obtain allantoic fluid, the blunt end of the egg was removed to expose the shell membrane, which was pierced with a blunt forcep. With a deliberate thrust, the allantoic sac was punctured. A sterile pipette or syringe and needle made it possible to aspirate the allantoic fluid. Care was taken to avoid rupture of the amniotic sac, the yolk sac, and blood vessels. It was possible to obtain clear allantoic fluid by this technic without previously chilling the eggs. *Erythrocyte suspension.* Hemagglutination tests were made with chicken erythrocytes that had been washed 3 times with phosphate buffered saline (pH 7.4). A 0.5% suspension of washed cells was prepared in buffered saline (pH 7.4). Freshly drawn red blood cells were used in each experiment. *Hemagglutination tests.* The hemagglutination test employed was a modification of the technic described by Salk(13). Titrations were made in round-bottom tubes measuring 7 x 75 mm. Doubling dilutions of allantoic fluid were prepared in buffered saline (pH 7.4) in such a manner as to leave 0.5 ml of the diluted virus in each tube. To each tube was added 0.5 ml of 0.5% washed chicken red blood cells. The titrations were incubated at room temperature for 1 hr and then read according to the pattern of the sedimented cells. The incubation time of 1 hr appeared to give the most satisfactory results. If allowed to stand longer, the pattern of the cells became indistinct and difficult to read. Diffuse agglutination patterns were interpreted as positive hemagglutinations while the appearance of a well defined "button" was considered as negative.

Experimental. A. *Growth of Newcastle disease virus as measured by infectivity titers.* Embryonated eggs, 12 days old, were inoculated via the allantoic sac with 0.2 ml of allantoic fluid containing approximately 50 LD_{50} of virus as determined by fertile egg titration. The dilution of this inoculum by the allantoic fluid contained in the egg was not determined. At intervals of 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 24 hours after infection 5 eggs were opened and 1.0 ml of allantoic fluid was removed from each egg and pooled. Doubling dilutions of each of the infected fluids were later inoculated into sixteen 10-day-old fertile eggs (4 eggs per each dilution) and their LD_{50} titers were determined by the method of Reed and Muench(14). Six inoculated eggs were allowed to incubate for a longer period and none of these died before 48 hours. Fig. 1 is a graphic presentation of the data obtained from two independent determinations.

It can be seen that between one and 4 hours after inoculation there was a relatively constant quantity of virus present in the allantoic fluid. Beginning at 4 hr there was a very sudden rise in the virus content which appeared to reach its maximum at 6 hr, after which there was a slackening in the output but not an arrest of virus production. The virus titer continued to rise slowly, and reached another heightened period between ten and twelve hours. A very definite leveling off of detectable virus became evident at 16 hr and was apparently sustained for the re-

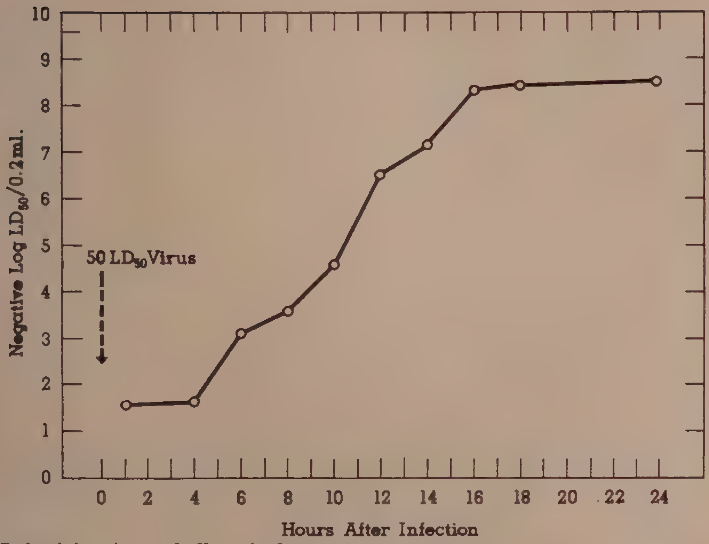

FIG. 1. Infectivity titers of allantoic fluid collected during a 24 hr period of the multiplication of Newcastle disease virus.

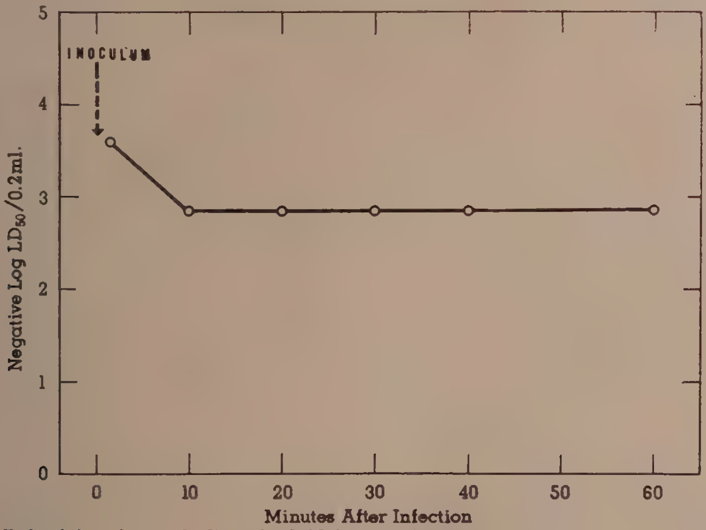

FIG. 2. Infectivity titers of allantoic fluid harvested during the first hour of infection with Newcastle disease virus.

mainder of the 24-hr period.

It was then decided to examine the growth activity of the virus immediately after its introduction into the egg. For this, 12-day-old, fertile eggs were inoculated with 0.2 cc of a 1/100 dilution of virus. This inoculum had been found to produce uniformly good infectivity. Fig. 2 contains the data obtained from the titrations of samples taken immediately and at 10 minute intervals. An examination of these data shows that a decrease in the virus content of the allantoic fluid takes place within 10 minutes after it has been introduced into the egg. The remaining 50

TABLE I. Infectivity and Hemagglutination Titers of Allantoic Fluid Collected During 1-24 Hr of Growth Period.

Sample (hr)	Infectivity titers*	Hemagglutination titer 1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
1	1.6	—							
2	1.5	—							
	2.5	—							
4	1.5	—							
	2	—							
6	3	—							
	3.5	—							
8	3.2	—							
	4	—							
10	4.3	—							
	5	—							
12	6	+							
	7	—							
14	6	+	+						
	8	+	+	+	+				
16	8.3	+	+	+	+				
	8.6	+	+	+	+	+	+		
18	8	+	+	+	+				
24	8.4	+	+	+	+	+	+	+	+
	8.6	+	+	+	+	+	+	+	+

* Infectivity titers expressed as negative logs.

minutes are marked by a stationary quantity of virus in the fluid.

B. *Detection of hemagglutinins during growth period of Newcastle disease virus.* Pooled samples of infected allantoic fluid harvested for infectivity titers were tested for their capacity to agglutinate washed chicken erythrocytes. Table I shows the results obtained in 2 determinations when doubling dilutions of these fluids were mixed with 0.5% red blood cells. Included in this table are the infectivity titers of these samples. It was not possible to demonstrate hemagglutination earlier than 12 hr following inoculation of virus into the egg. The first appearance of hemagglutination was followed by increased titers in the subsequent samples. Tests were not carried out beyond the 24-hour period. Similar determinations were made on samples of allantoic fluid collected every 10 minutes during the first hour of infection. None of the samples tested gave titers above 1:2 and in the 60 minute sample the presence of hemagglutination in the dilutions of 1:2 was doubtful. This reaction was not checked for non-specific hemagglutination.

C. *Effect of irradiated virus on multiplication of active Newcastle disease virus.* Twelve-day-old embryonated eggs, infected with 10 LD_{50} of Newcastle disease virus, were inoculated with 0.5 ml of undiluted allantoic fluid containing 250 LD_{50} of Newcastle disease virus that had been inactivated by exposure to ultra-violet irradiation for 9 minutes. The inactivated virus suspension was introduced into the egg 1 hr after the infective dose had been administered. At intervals of 2, 4, 6, 8, 10, and 12 hr following the infective dose, 5 eggs were opened and 1.0 ml of allantoic fluid from each egg was removed and pooled. A titration of viable virus was done by preparing dilutions of the pooled samples and inoculating 0.5 ml of the dilutions into four 10-day-old fertile eggs. The data for this experiment are presented in graphic form in Fig. 3. When this figure is compared with the growth curve of this same virus (Fig. 1) it can be seen that the addition of irradiated virus to embryonated eggs already infected with viable virus has the ability of altering the usual course of events. A step-like growth pattern was observed suggesting abrupt inter-

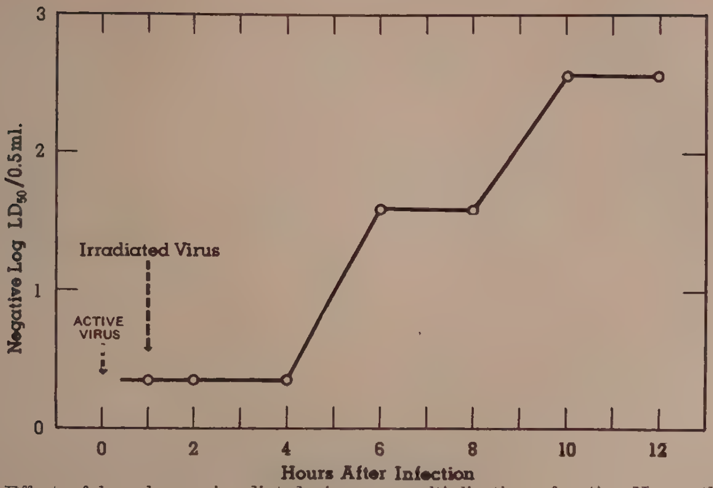

FIG. 3. Effect of homologous irradiated virus on multiplication of active Newcastle disease virus.

ruptions in growth.

Discussion. The findings in the foregoing experiments appear to indicate that Newcastle disease virus inoculated into the allantoic sac of the embryonated egg was adsorbed onto susceptible embryonic tissue before it began to proliferate. The introduction of a quantity of virus into the allantoic sac is followed by a static period of approximately 4 hr during which time a constant amount of virus can be detected in the allantoic fluid. Within 10 minutes after its introduction approximately 87% of the infective dose disappeared from the allantoic fluid (Fig. 2). It appears therefore that an equilibrium between virus in the allantoic fluid and virus in the cells of the chorio-allantoic membrane had been established during the first 10 minutes and maintained for the remainder of this 4 hr period. Similar findings have been reported by Stulberg, Schapira and Todd in tissue culture studies(15).

The constancy in quantity of virus recoverable from the allantoic fluid during the first 4 hr of infection would seem to indicate that the virus activity in progress is occurring within susceptible host cells, and does not influence the infectivity titers of allantoic fluids examined during this period. Presumably the virus present in the allantoic fluid during this phase of apparent quiesence represents virus that had been prevented from entering susceptible cells.

At the conclusion of the 4th hr of infection, there is a very marked increase in the quantity of virus in the allantoic fluid. Stulberg and his co-workers observed this increase to occur between 9 and 11 hr(15).

Employing the technic for producing an interference phenomenon, an attempt was made to block the advent of a second growth cycle. While the results as presented in Fig. 3 are equivocal, they are suggestive of a growth cycle of approximately 4 hr duration. Assuming the first growth cycle to end 4 hr after its initiation, the marked increase of virus in the allantoic fluid that follows may be the result of the elution of multiplied virus from chorioallantoic cells. The plateau in the curve between 6 and 8 hr can then be thought to be a period of maximum adsorption of active virus onto susceptible cells during the second growth cycle. The inactivated virus presumably reduced the number of potentially susceptible cells and thus there was a limitation of adsorption as suggested in Fig. 3. Most significant, however, is the rise in virus titer at the 8th hr after the proposed "first burst."

The inability to demonstrate hemagglutinating titers compatible with infectivity titers during the early phase of growth may be explained by either assuming that a hemagglutinating unit of virus had not yet been accumulated or by the alternate hypothesis offered by Henle and Henle(4) who suggest that the formation of influenza virus proceeds through a development of immature forms or building block. The contentions of these workers can be extended to the present study since it can be seen that while the infectivity titers of the 16th, 18th and 24th hr samples remain stationary, the hemagglutinating titers continue to rise. This paradox almost bespeaks a maturation process that endows the virus with its hemagglutination property, and is an event which lags behind the development of its infectivity.

Summary. 1. Data are presented to support the contention that the growth of Newcastle disease virus in the developing chick embryo occurs in cycles of 4 hr. The apparent 4-hr latent period in the production of virus following the inoculation of an infective dose does not correspond to the lag phase in the multiplication of bacterial cells, but rather indicates an intercellular development of virus particles that cannot be determined by any of the present technics for measuring viral activity. The maximum adsorption of virus onto susceptible cells occurs within 10 minutes after its introduction into the embryonated egg. 2. The existence of an unequal development of infective and hemagglutinating titers during the growth of Newcastle disease virus is described and an attempted explanation for this discrepancy is made on the hypothesis that the hemagglutination unit results from a maturation of the infectious particles and cannot therefore be detected early in its growth.

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Response of the Baby Chick to West Nile Virus. (19572)

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The virus employed in this study was the B 956 strain of West Nile virus furnished by the American Type Culture Collection in Washington, D. C. This virus was obtained by the above institution from Dr. K. C. Smithburn(1) of the Rockefeller Foundation Laboratories in New York, who had isolated this virus strain from a patient in Uganda, Africa and had passed this virus 25 times in Swiss albino mice by intracerebral inoculation. This virus has been cultivated in chick embryos by other investigators(2). At this laboratory the above-mentioned virus was passed 4 additional times in mice by the intracerebral route before initiating the present study.

Methods and results. Three-week-old Swiss albino mice (Webster strain) were employed.

This strain of mice was the one recommended by Webster(3) as being especially susceptible to neurotropic viruses. The baby chicks employed were 1-day-old and were furnished by a local farm in the state of Maryland.

Each of 6 mice were inoculated intracerebrally with 0.03 ml of a 10% suspension of West Nile virus of the 29th intracerebral mouse passage. The mice showed involuntary motor reactions on the 4th day. When these symptoms of central nervous system involvement appeared, the mice were sacrificed and the brains were removed aseptically. The pool of infected mice brains was ground with alundum and diluted to a 10% suspension with physiological saline. The suspension was then subjected to 5 minutes centrifugation in a horizontal centrifuge at 2,000 r.p.m. The supernatant was used as the inoculum for initiating the virus experiment in baby chicks. The virus titrated 10^{-6} in Swiss albino mice inoculated intracerebrally. The supernatant material was injected intracerebrally into 6 unvaccinated mice and into 6 mice which had been immunized previously with the 25th mouse passage of West Nile virus. Immunization was accomplished by intramuscular injections using high dilutions of the virus over a period of several weeks. After 6 days the unvaccinated mice showed symptoms of central nervous system involvement, while the immunized mice showed no nervous symptoms during a 30-day observation period. This confirmed the virus to be West Nile virus.

Six 1-day-old baby chicks were injected in the right frontal lobe with 0.05 ml of the supernatant from the virus-bearing mouse brain suspension, and 6 Swiss albino mice were injected intracerebrally with 0.03 ml of the same suspension. From 3 to 6 days after injection the baby chicks showed irritability, extreme nervous tremors, and paralysis. When nervous symptoms developed, the brains were removed aseptically, ground with alundum and diluted to a 10% suspension with physiological saline. This suspension was passed to 6 baby chicks and 6 Swiss albino mice by intracerebral injection. The mice showed West Nile nervous symptoms between 6 and 8 days after inoculation and were discarded. The West Nile virus was carried serially through 8 passages in the baby chick by intracerebral inoculation as shown in Table I. A 10% suspension of virus-bearing chick brain from each passage was injected intracerebrally into 6 Swiss albino mice in order to determine the presence of West Nile virus. All of the mice succumbed to the disease. The incubation periods are shown in Table I. Three hundredth ml of a 10% suspension of virus-bearing chick brain of the 8th intracerebral passage was injected intracerebrally into 6 unvaccinated mice and into 6 mice which previously had been immunized with the 25th mouse passage of West Nile virus. Immunization was accomplished as previously described. After 6 days the unvaccinated mice showed symptoms of central nervous involvement, while the immunized mice showed no nervous symptoms during a 30-day observation period. This confirmed the virus in the brain material of the 8th intracerebral chick passage to be West Nile virus. Throughout the 8 intracerebral baby chick passages, the chicks showed typical symptoms such as paralysis, nervous tremors, malaise, and death.

TABLE I. Response of Baby Chicks and Swiss Albino Mice Inoculated Intracerebrally with West Nile Virus.

Passages	No. chicks exposed	No. chicks showing symptoms	Min to max incubation period,* days	
			Chickens	Mice
1	6	6	3-6	6-8
2	6	4	3-5	6-8
3	6	4	3-5	3-5
4	12	12	4-6	3-5
5				
6				
7				
8				

* Incubation period = time between exposure to virus and onset of symptoms.

Twenty-eight healthy 1-day-old baby chicks were divided into 7 groups of 4 chicks each. Chicks of the first 5 groups were administered 0.06 ml of a 10% suspension of the virus-bearing chick brain of the 7th intracerebral baby chick passage by one of the following methods: intraperitoneal, intradermal, intramuscular, intralingual, and intracardiac injections. Five-hundredths ml of the same

TABLE II. Response of Baby Chicks to West Nile Virus of 7th Intracerebral Chick Passage.

Route of inoculation	No. chicks exposed	No. chicks showing symptoms	Min to max* incubation period, days
Intraperitoneal	4	3	5–10
Rectal	4	4	
Intradermal	4	3	
Intracardiac	4	3	
Intramuscular	4	4	
Intralingual	4	4	
Intranasal	4	4	

* Incubation period = time between exposure and onset of symptoms.

inoculum was instilled intranasally in chicks of the 6th group and rectally in chicks of the 7th group.

After inoculation all groups were observed twice daily for nervous symptoms characteristically found in baby chicks infected experimentally with West Nile virus. The results of this group exposure are given in Table II.

Summary. A strain of West Nile virus isolated by Dr. K. C. Smithburn from a patient's serum in Africa and passaged intracerebrally in Swiss albino mice has been successfully transmitted intracerebrally to 1-day-old White Leghorn chicks. The virus was carried intracerebrally through 8 serial passages in 1-day-old baby chicks. Virus-bearing brain suspensions from chicks of each passage proved to be infective for Swiss albino mice when inoculated intracerebrally. Virus from a chick-brain suspension of the 7th intracerebral passage was transmitted successfully to baby chicks by the following routes of exposure: intraperitoneal, intradermal, intramuscular, intracardiac, intralingual, intranasal and rectal. Baby chicks showed typical symptoms of West Nile disease between 5 to 10 days post-inoculation.

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Further Studies on Antigenic Variation of Influenza A Virus. (19573)

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In a previous paper(1) it was shown that antigenic variants of influenza A virus strains emerge during serial passage *in ovo* in the presence of immune serum against antigenically different but related strains. It was also demonstrated that the modified strains retained their new antigenic patterns in the absence of immune serum.

It was of interest to determine how long the new antigenic properties would persist on serial passage in the absence of the selective environment used to obtain the variants. This report is concerned with the latter problem.

Materials and methods. Viruses. Two strains of influenza A virus, PR8 and 965, and the variants obtained from them on passage with heterologous immune serum(1) were used. The variant strains had been through 6 passages in the absence of immune serum before the present experiments were begun. In the experimental section, the lines of a particular strain, *e.g.*, PR8, are identified by subscripts as follows: original strain = $PR8_o$; control passage strain = $PR8_c$; heterologous immune serum passage strain = $PR8_9$ (subscript corresponding to the strain, *i.e.*, 965, used to produce the immune serum). Virus titrations, immune serum preparation, neutralizing antibody titrations, and hemagglutination-inhibiting antibody titrations were carried out exactly as in the previous study(1). In the serial passages, the inoculum consisted of 0.2 cc of a 10^{-3} dilution of infected allantoic fluid.

Experimental. The variant strains $PR8_9$ and 965_p which had been through 6 serial passages *in ovo* in the presence of the heterologous immune serum, and then through 6

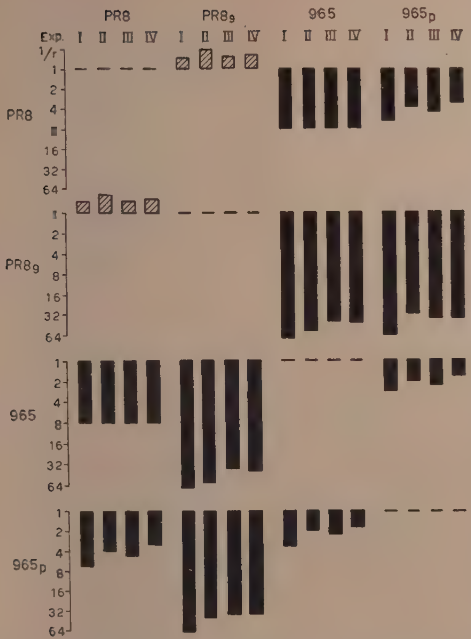

FIG. 1. Extent of cross-neutralization reactions with PR8, 965 and variants derived from them on serial passage *in ovo* in presence of heterologous immune serum. Exp. I, with immune serum; Exp. II, III and IV represent 6, 12, and 24, respectively, additional passages without serum.

additional serial passages in the absence of immune serum, were studied further. In the present study they were subjected to a total of 18 further passages *in ovo* in the absence of immune serum. Cross-serological experiments, by both the *in ovo* neutralization and hemagglutination-inhibition procedures, were carried out at the 6th and 18th additional passages. The objective was to determine if the new antigenic patterns which had appeared on passage in the presence of immune serum would persist throughout a total of 24 *in ovo* passages without serum.

The results of complete cross-neutralization reactions with PR8, 965 and the variant strains derived from them are shown in Fig. 1. The findings in 4 successive experiments during the passage series are illustrated for purposes of comparison. The data for Exp. I and II are taken from the earlier paper of Archetti and Horsfall(1); those for Exp. III and IV were obtained in the present study. The ratio r represents the geometric mean value of the 2 heterologous titer ratios (r_1 and r_2) and was determined by means of the equation $r = \sqrt{r_1 \times r_2}$(1). In Fig. 1, the ratio r is shown as the reciprocal, $1/r$, and the extent of the antigenic difference between strains is indicated by the length of the blocks. Exp. I was carried out after 6 passages in the presence of immune serum; Exp. II after 6 additional passages without serum(1). Exp. III and IV were done after 12 and 24 passages, respectively, without serum. In all experiments, the same sera were used in each of the cross-neutralization reactions. These were prepared against the strains obtained in Exp. I(1).

It is clear from the results shown in Fig. 1 that the values of $1/r$ found in Exp. III and IV were not significantly different from those obtained in Exp. I and II. This evidence indicated that the antigenic differences, demonstrable by the neutralization procedure, which appeared after passage in the presence of immune serum, tended to persist through a series of 24 *in ovo* passages without serum.

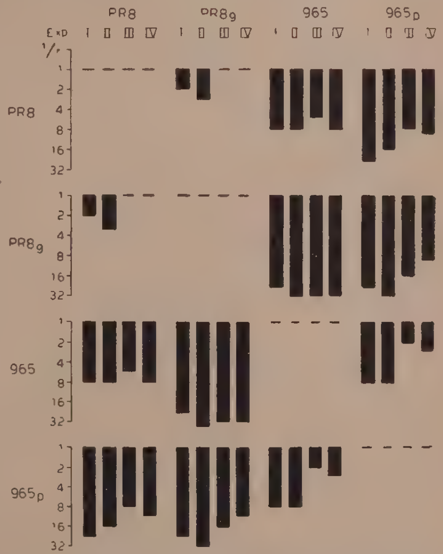

FIG. 2. Extent of cross-hemagglutination-inhibition reactions with PR8, 965 and variants derived from them on serial passage *in ovo* in presence of heterologous immune serum. Exp. I, with immune serum; Exp. II, III and IV represent 6, 12, and 24, respectively, additional passages without serum.

The results of cross-hemagglutination-inhibition reactions between PR8, 965 and the variant strains derived from them on passage with heterologous immune serum are shown in Fig. 2. For comparison, the results of 4 successive experiments during the passage series are presented. As in the case of Fig. 1, the data for Exp. I and II are taken from a previous paper(1), whereas those for Exp. III and IV derive from this investigation. The passage history of the strains employed in Exp. I to IV is described above. The sera were identical with those used in the cross-neutralization reactions.

It appears from the data shown in Fig. 2 that the antigenic differences between PR8 and $PR8_9$ disappeared after 12 passages in the absence of immune serum. However, the differences between 965 and 965_p remained demonstrable, by the *in vitro* procedure, through 24 passages without serum although they were less marked than after 6 such passages. This evidence supports that obtained in the cross-neutralization experiments.

Discussion. It was demonstrated previously that it is possible to select antigenic variants of influenza A virus strains by passage *in ovo* in the presence of immune serum against a somewhat different strain. The new antigenic patterns persisted for 6 serial passages in the absence of immune serum(1). On increasing the number of serial passages without serum to a total of 24, the antigenic differences between one parent strain (965) and its variant persisted, while those between the other parent strain (PR8) and its variant disappeared. The variant (965_p) which showed the persistence of antigenic properties different from those of the parent strain was that which manifested such differences both in neutralization and in hemagglutination-inhibition experiments. On the other hand, the variant ($PR8_9$) which appeared to revert on prolonged passage without serum was that which had shown antigenic dissimilarity only in hemagglutination-inhibition experiments.

Summary. Antigenic differences in influenza A virus strains which appear during serial passage *in ovo* in the presence of heterologous immune serum may remain demonstrable for at least 24 passages without serum.

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Pharmacological Studies on Irradiated Animals. II. Effect of Cortisone on X-Ray Mortality of Mice.* (19574)

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In continuation of our systematic pharmacological studies on irradiated animals, the effect of cortisone on the lethal effect produced by X-rays in total body irradiation of mice is here reported.

Materials and methods. Swiss male white mice of the Institute strain, 22 g ± 15% of body weight, were used. Irradiation was done as described previously(1). The radiation factors were: 200 kv, 15 ma, .25 Cu + 1.0 mm Al filtration, HVL = .8 mm Cu. In agreement with previously outlined rules for pharmacological studies on irradiated animals (2), the effect of a standard dose of cortisone of .25 mg (Cortone (Merck) 1 cc = 25 mg) was administered intramuscularly to mice exposed to various X-ray doses namely those of 410, 350, and 200 r/air, representing the LD 60 and 25/14 days and minimal lethal dose, respectively. Hormone injections were given immediately after exposure and continued to the 14th day after irradiation. For graphic

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* The opinions or assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

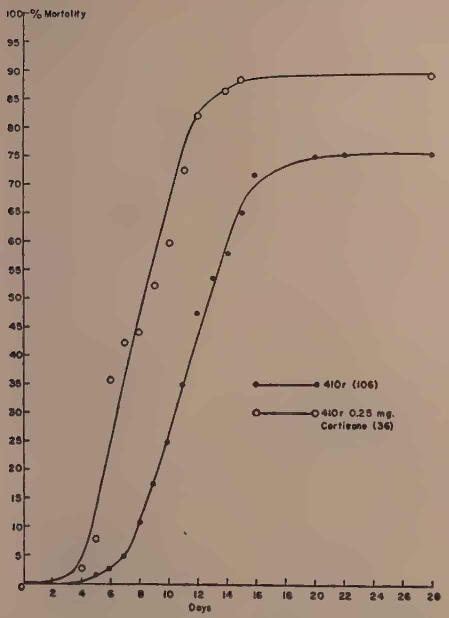

FIG. 1. Comparison of percentage mortality in mice irradiated with 410 r, total body irradiation; cortisone-treated group (open circles) and non-medicated group (solid circles).

presentation of results the mortality rate of hormone treated animals is compared with that of the non-medicated irradiated control group. Figures in parenthesis indicate the numbers of animals in the different groups.

Results. 1. *Effect of cortisone on radiation induced mortality.* Fig. 1 and 2 show the influence of cortisone on the mortality rates produced by the 3 X-ray doses. At all 3 doses death started earlier in the cortisone-treated and irradiated animals than in their non-medicated irradiated controls. There was a definite increase in the mortality rates produced by the use of cortisone in animals exposed to the X-ray doses of 410 and 350 r respectively. These differences in mortality rate are statistically significant at the end of the first week after irradiation ($P < 0.01$ for the 6th day for 410 r, and $P = 0.02$ for the 7th day for 350 r) although not throughout the entire period.[†] No increase in mortality was noted at 200 r. (Fig. 2B.)

2. *Effect of cortisone and ascorbic acid treatment on the lethal effect of total body irradiation.* In view of the demonstrated increase of the radiation induced mortality rate of total body irradiation by cortisone administration, an attempt was made to ascertain whether additional treatment with ascorbic acid might influence the cortisone effect on irradiated mice. This investigation was based on the premise that cortisone in large doses affects the blood forming tissues similarly to X-rays (3,4). There are reports indicating that large amounts of ascorbic acid speed up the restoration of the white blood count after x-irradiation(5) and also after benzol administration (6). Even though ascorbic acid seems not to influence radiation induced mortality(7), it was thought that this substance might modify the influence of cortisone on the radiation effects. Consequently, two sets of experiments were carried out to test this hypothesis. Animals exposed to doses of 410 and 200 r/air received in addition to the standard dose of .25 mg cortisone, daily intramuscular injections of 15 and 5 mg of

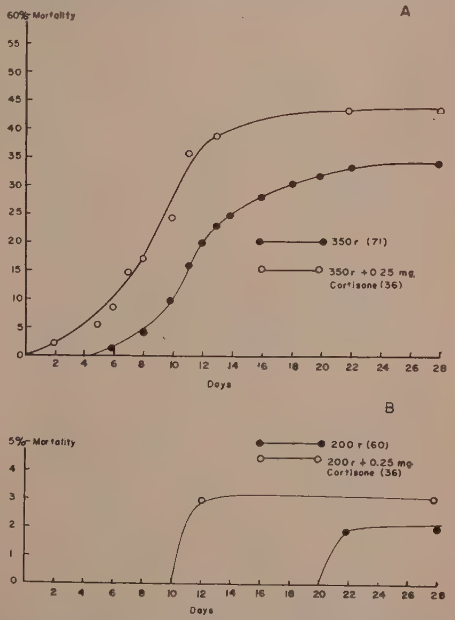

FIG. 2. Comparison of percentage mortality in mice irradiated: A, with 350 r and B, with 200 r, total body irradiation; cortisone-treated group (open circles) and non-medicated group.

[†] We are indebted to Dr. J. P. Flynn for the statistical analysis.

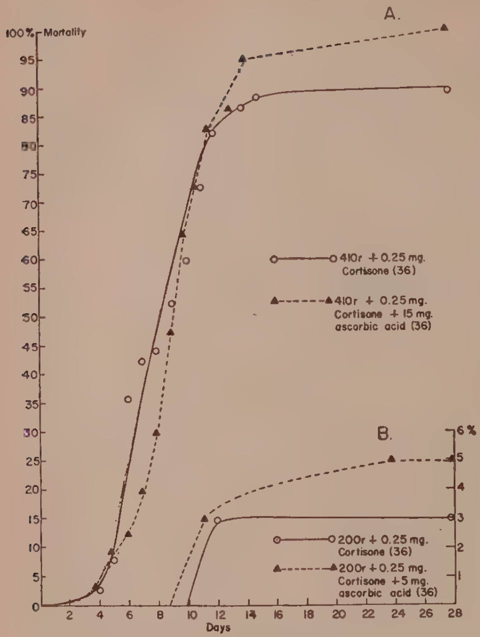

FIG. 3. Comparison of percentage mortality in mice irradiated: A, with 410 r and B, with 200 r, total body irradiation; cortisone-treated group (solid line), cortisone and ascorbic acid-treated group (broken line).

ascorbic acid respectively for 14 days after irradiation. The pertinent results summarized in Fig. 3A and B indicate that administration of ascorbic acid in the doses used did not change significantly the course of the cortisone effect.

Discussion. Our results concerning the effect of cortisone administration on the lethal effect of total body irradiation with X-rays clearly indicate an enhancement of the radiation induced mortality in this animal species. The effect was most pronounced at the end of the first post-irradiation week. These data confirm and supplement previous observations of Smith, Smith, and Thompson(8) who noticed "failure of cortisone or ACTH to reduce mortality in irradiated mice." Our observations also emphasize the suggested procedure in pharmacological studies on irradiated animals(2), to use a standard dose of a pharmacological agent on at least 2, preferably 3, X-ray dose levels in order to clarify more completely such drug action. The enhancement of the effect of total body irradiation by cortisone, in contra-distinction to the previously reported beneficial effects of DCA (9), is not surprising on the basis of the known physiological actions of these two substances. Edelmann(10) recently demonstrated that not only DCA but also whole extract of the adrenal cortex has a beneficial effect on radiation induced mortality of adrenalectomized rats. This seems to indicate that the effects of cortisone within the gland may be balanced by others of the numerous substances elaborated by the adrenal cortex. Thus the effect of irradiation on the adrenal cortex may well consist not only in stimulation and eventual exhaustion of secretion, but also result in a disturbance of the balance of these various adrenal cortical hormones in the secretion.

The unfavorable effect of cortisone on irradiated animals appears noteworthy from a clinical point of view: the simultaneous use of cortisone and ionizing irradiation as well as the use of this substance shortly after an acute exposure to this type of radiant energy must be considered contra-indicated.

Summary. 1. Administration of 0.25 mg cortisone intramuscularly to mice daily for 14 days after their exposures to the LD 60 and 25/14 days of x-rays resulted in a definite increase of the mortality rates produced by these x-ray doses. 2. There was no increase in mortality in the cortisone-treated mice exposed to the minimal lethal dose of x-rays but as in the higher x-ray doses, cortisone treatment produced a shortening of the latent period between irradiation and onset of death, indicating an adverse effect even at this low radiation level. 3. Simultaneous treatment of irradiated mice with cortisone and ascorbic acid did not modify the enhancement of the radiation induced mortality rate produced by the cortisone administration. 4. Some clinical implications of these animal experimental data are briefly mentioned.

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Phage Multiplication on Two Hosts. Isolation and Activity of Variants of Staphylococcus Phage P_1.* (19575)

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By simultaneously titrating phage on two susceptible hosts, the authors have been able to detect and isolate variants from a stock staphylococcus phage P_1. The dual host assay system has provided a sensitive means of studying certain unusual activities of one of the phage variants.

Experimental procedures and results. I. *Isolation of phage variants from stock P_1.* When aliquots of stock P_1, prepared on host strain Staphylococcus K_1 in tryptose-phosphate both, were titrated by plaque count(1) on two hosts—strains K_1 and WF 145‡—a slightly but consistently higher titration value was found with the latter. This difference could not be ascribed to rates of adsorption, latent periods, or burst sizes of the phage for the two hosts. Stock phage P_1 was then produced on strain 145 in trypticase soy broth. At intervals aliquot samples were titrated on the two hosts. The ratio of titer per ml on 145 to titer per ml on strain K_1 (hereafter designated $145/K_1$) was found to increase to approximately 4 during the growth period of the phage. Plaque isolates were made from samples exhibiting this high ratio. Virus from plaques on strain 145 was suspended in saline and aliquots plated on the two hosts. Four isolates of 32 exhibited distinct differences in titration ratio $145/K_1$, and, in 2 cases, formed small sized plaques on K_1 cells. Growth in broth on 145 cells resulted in characteristic curves of phage titratable on the two hosts. Two variants, No. 15 and 16, showed very similar curves and titration ratios $145/K_1 =$ ca 5. Since they also formed small plaques on K_1 cells, they were considered identical isolates. One variant, No. 5, exhibited a growth curve much like that of stock phage and proved to have a titration ratio of 1. The lysate of the fourth, phage 14, exhibited a ratio $145/K_1 =$ ca 40. This variant was selected for detailed investigation to determine the factors involved in producing this high ratio.

II. *Observations on phage 14.* A. *Attempted strain separation by plaque isolation.* The high plaque ratio $145/K_1$ of P_{14} lysates of 145 cells pointed to a possible mixture of virus strains, one of which produces no plaques on K_1 assay cells. A series of 10 successive serial passages of P_{14} on host K_1 was made by plating saline suspensions of virus from individual plaques. On the tenth transfer the saline suspension of virus assayed on the 2 hosts in a ratio $145/K_1 = 1.3$. A similar

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† Edith Claypole Memorial fellow in Bacteriology.

‡ Obtained through the courtesy of Dr. John E. Blair, New York Hospital for Joint Diseases.

TABLE I. Effect of Host Passage on Titration Ratio $145/K_1$ of Phage Variants.

Variant No.	Plaque formation on K_1 cells	Avg value $R145/K_1$ of virus suspended from plaques, after: 10 passages by plaque transfer on Host K_1	Host 145	1st transfer from K_1 to Host 145
5	Indistinguishable from Stock P_1	1	1	
14	"	1.3	40	40
15	Tiny	1	7	

series of 10 passages was carried out on strain 145. (Hereafter, P_{14} which has been carried on K_1 cells will be referred to as $P_{14}(K_1)$, and that passaged on strain 145 as $P_{14}(145)$. After 10 serial passages, virus from plaques on strain 145 assayed in a high ratio, suggesting that a separation of at least 2 strains had been made. However, when virus which had been carried on K_1 cells was transferred back to host 145, it produced plaques whose contents assayed again in a high ratio. This return to a high ratio occurred in the first passage and showed that no separation had been achieved. Two other variants, No. 5 and 15, were also passaged on each host. One of them, phage 5, maintained a ratio of one independent of the host used for passage, indicating that the shift in ratio occurring with P_{14} is a property peculiar to this variant. Representative data are summarized in Table I.

B. *Titration ratios of P_{14} in adsorption and multiplication studies.* 1) *Production in broth on 2 hosts.* P_{14} infections of log phase 145 cells in Broth III (3% tryptose phosphate, 3% trypticase soy, and 0.5% yeast extract) invariably produced a clear lysate with a high ratio $145/K_1$, whereas on K_1 cells, the phage in the lysate assayed in a ratio approaching one. This occurred regardless of which host the phage had previously been passaged on. The ratios are characteristic for assays of free phage and correspond to similar assays of saline suspensions of virus obtained from plaques in the same medium.

2) *Adsorption on two hosts.* Phage 14 was adsorbed on both 145 and K_1 cells in broth III for 20 minutes at 37°C, in a ratio of phage/cells = ½. At the end of this period, samples were centrifuged and resuspended to volume in broth III, to collect infected cells; and were filtered through super cell pads, to obtain the unadsorbed free phage. Dual assays were run on 1) total phage, 2) infected cells, and 3) free phage. As summarized in Table II, when $P_{14}(K_1)$ was added to K_1 cells or to 145 cells, 98-99% of the phage was absorbed in 20 minutes. In both cases, the residual free phage assayed in a ratio $145/K_1 = 1$. The infected cells (both K_1 and 145) also assayed in a ratio of 1, and the absolute titer corresponded to the value of the total free phage on each host. When $P_{14}(145)$ was added to K_1 or to 145 cells, about 99% of the phage was adsorbed in 20 minutes. In both cases ($P_{14}(145)$ plus K_1 cells and $P_{14}(145)$ plus 145 cells), the residual free phage assayed in the high ratio characteristic of dual assays of the total free phage. The infected K_1 and 145 cells showed assay ratios $145/K_1 = 1$ when aliquots of each were titrated on the two hosts. However, the absolute titer per ml of the infected K_1 cells was reduced to the titer exhibited by total free $P_{14}(145)$ on host K_1, whereas the titer of the infected 145 cells corresponded to the total free phage as measured on 145 cells. Since 99% of the phage had been adsorbed onto the K_1 cells, and since the titer of infected cells was very low, these results show that a high percentage of adsorbed particles produced no plaques on host K_1. Furthermore, this same percentage of "infected" K_1 cells release no phage capable of forming plaques on host 145. On the other hand, when the same quantity of phage 14(145) is

adsorbed on strain 145, most or all of these infected cells are capable of forming plaques in both K_1 and 145 assay suspensions. Thus, the high ratio $145/K_1$ of free phage 14(145) is not the result of great differences in the amount of adsorption onto the two hosts, but is apparently related to the release of phage from "infected" cells. 3) *Latent periods and Burst sizes of P_{14} on the two hosts.* The latent period of $P_{14}(145)$ added to either host K_1 or host 145 log phase cells was found to be around 30 minutes at 37°C. This value was independent of the strain used to assay the rise in titer. Tests on $14(K_1)$ show a similar latent period on the two hosts.

Log phase K_1 cells infected with 14(145) released about 60 particles per infected cell when aliquots were assayed on strain K_1, and 75 particles per infected cell when assayed on strain 145. Similar results were obtained with $P_{14}(K_1)$ infections of K_1 cells. This showed that phage released in the first burst from infected K_1 cells reproduces the assay ratio of ca 1.3 characteristic of broth lysates of strain K_1. A log phase infected Staph. 145 cell was found to release by burst only 2 to 3 particles capable of forming plaques on host K_1 as compared with 80 particles capable of producing plaques on host 145. Thus, analyses of phage produced in the first burst showed an assay ratio $145/K_1$ of ca 40. Again the burst size was independent of the host used to passage the virus P_{14}. *Viz.* Table II.

C. *Tests for inhibitors.* The fact that most of the phage particles produced by host 145 adsorb onto K_1 cells but do not form plaques suggested the possibility of an inhibitor for K_1 lysis associated with phage 14 and strain 145. 1) Assays of filtered phage from P_{14} multiplying on host 145 showed a consistently high ratio $145/K_1$, indicating that free phage early in production is no different than that released in the last bursts, and that the high ratio does not result from an accumulation of an inactivator. 2) Filtrates from log phase normal K_1 or 145 cells produced no inhibitory effect on bursts of phage from strain 145. 3) $P_{14}(K_1)$ was mixed with $P_{14}(145)$ and stored at 4°C overnight. The total count was equal to the sum of controls mixed similarly with

TABLE II. Summary of Titration Ratios in Adsorption and Burst Size Studies.

Phage and passage on cells	Phage assay on cells	Plaque suspension after 10 passages/ml*	Total phage in filtered broth lysate per ml†	Adsorbed on K_1 cells 20 min 37°C: Residual free phage Per ml‡	Residual free phage % adsorbed	Infected cells per ml§	Burst size per infected cell‖	Adsorbed on WF145 cells 20 min 37°C: Residual free phage Per ml	Residual free phage % adsorbed	Infected cells per ml	Burst size per infected cell
$14(K_1)$	K_1	1.1×10^6	$1\ \times10^8$	1.1×10^6	98.9	5.6×10^7	100	1.6×10^6	98.4	4.4×10^7	2
	145	1.4×10^6	1.4×10^8	1.3×10^6	99.1	5.8×10^7	100	2.5×10^6	98.2	5.6×10^7	60
	$\frac{R145}{K_1}$	1.3	1.4	1.2	—	1	1	1.5	—	1.2	30
14 (145)	K_1	$5\ \times10^4$	$2\ \times10^6$	1.1×10^4	99.4	1.3×10^6	60	2.1×10^4	99	3.6×10^7	2
	145	2.2×10^6	6.8×10^7	4.7×10^5	99.3	1.5×10^6	75	6.8×10^5	99	5.6×10^7	83
	$\frac{R145}{K_1}$	40	34	42	—	1.2	1.3	33	—	1.5	42
Stock $P_1(K_1)$	K_1	—	6.9×10^7	5.2×10^5	99.2	6.2×10^7	80	5.1×10^5	99.3	6.6×10^7	58
	145	—	9.4×10^7	6.2×10^5	99.3	$6\ \times10^7$	85	6.5×10^5	99.3	7.2×10^7	62
	$\frac{R145}{K_1}$	—	1.4	1.2	—	1	1.1	1.3	—	1.1	1.1

* Ten ml saline suspension of virus from plaque after 10th passage. † Log phase cells infected with phage in Broth III, incubated 37°C until lysis. Filtered through super cell. Diluted in Broth III. ‡ Adsorption mixture $P/B=1\times10^8/2\times10^8$ per ml. Shaken 20 min 37°C, chilled ½ min, filtered through super cell. § Adsorption mixture $P/B=1\times10^8/2\times10^8$ per ml. Shaken 20 min 37°C, chilled ½ min, centrifuged in cold. Cells resuspended to volume. Some cells are lost in supernate. Recovery is about 50 to 75% of total cells. ‖ Unit infection of log phase cells $P/B=3\times10^8/1\times10^8$. Adsorbed at 37°C, free phage removed by centrifugation or determined by filtration of chilled sample through super cell. Infected cells diluted to *ca.* 10000/ml to prevent readsorption of phage. Average values reported.

broth. The mixture was additive for assay on K_1 and on 145 cells. The results were the same with phage mixed in dilute or in concentrated suspension. 4) Supernates from P_{14} (K_1) and P_{14}(145) were crossed after high speed centrifugation. Phage resuspended in these supernates was equal in titer to controls which had been centrifuged and resuspended in their own supernate. The above tests indicated that there was no interaction of phage lots passaged on different hosts. 5) Heat inactivation of P_{14}. Lots of $P_{14}(K_1)$ and P_{14}(145) were heated to 60°C. Dual titrations of samples removed at intervals revealed that activity for the K_1 cells decreased more rapidly than that for the 145 strain. Finally samples were obtained which had no phage active on K_1 but which exhibited residual activity for 145 cells. When fresh phage was diluted with these heated samples and exposed to 59°C, its activity decreased at rates similar to a control containing fresh phage diluted in broth, indicating that neither had anything heat stable been released nor had an inactivator been destroyed during heat treatment.

Examination of the last survivors of heat inactivation by plaque isolation from growth on 145 cells showed that all 47 isolated produced plaques both on K_1 and 145 cells. This occurred regardless of previous host passage. The ratio $145/K_1$ obtained by plating aliquots of plaque suspensions again approached 40.

Summary. Analysis of phage activity by titration on two hosts has revealed the presence of variants hitherto undetected in stock phage P_1. One isolate, Phage 14, exhibits great changes in titration ratio on passage through the two hosts K_1 and WF 145. Produced on K_1 cells, the ratio of free phage assayed on two hosts is 1.3, whereas made on 145 cells, the free phage titrates in a ratio $145/K_1$ of ca 40. This occurs regardless of the host employed in previous passage and is reproduced in the very first burst from infected cells. Attempts to isolate different strains from this phage by usual plaque isolation technics were not successful. The high ratio obtained with free phage made on 145 cells could not be explained on the basis of differences in adsorption onto or latent periods in the two hosts. The difference was traced to the production of a large number of particles from host 145 which adsorbed on K_1 but formed no plaques. No evidence was found for an inhibitor of K_1 activity associated with phage 14 production on 145 cells. Heat inactivation destroyed phage activity for K_1 cells much more rapidly than for 145 cells. There was no interaction of heat killed and active phage on exposure to 59°C.

Evidence has been accumulated which indicates that the phage P_{14} is altered on passage through host 145. The fact that phage particles surviving a heat treatment which destroyed all activity for K_1 cells produce on strain 145 a mixture of two phages (one active on K_1 and one—or both—active on strain 145) points to an unusual host effect on virus reproduction.

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Soluble Specific Leptospiral Complement-Fixing Antigens. (19576)

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Leptospiral complement-fixing antigens have been prepared from suspensions of the organisms by various methods in several laboratories (1-4). Carlinfanti(5) reported a "lipoidal" antigen extracted from washed, dried leptospirae. These antigens are characterized by relatively broad spectra of reactivity with antibodies against heterologous leptospiral strains.

This paper reports the demonstration of

soluble, highly strain specific antigens formed by leptospirae propagated on artificial mediums.

Materials and methods. Type strains of leptospirae, antigenically distinguishable by means of cross agglutination-lysis after Schüffner and Mochtar(6) were propagated in Stuart's(7) liquid medium. Cultures were incubated at 30°C for periods of 10 to 14 days. An aqueous solution of merthiolate, to a final concentration of 1:10,000, was added to the whole cultures to kill the leptospirae and to act as a preservative.

Leptospirae were removed from the culture liquor by centrifugation at 20000 g for 10 minutes at 5°C in a PR-1 International refrigerated centrifuge. The supernatant culture liquor was then filtered through a Seitz E-K pad. The organisms collected by centrifugation were resuspended in phosphate buffered saline solution at pH 7.2 to one-tenth the original culture volume. The organism suspension was exposed to vibration at 9400 cycles/second in a Raytheon Magnetostricton vibrator for 10 minutes, to prepare antigens of the sonic-vibrated type(4). In preliminary experiments, aliquots of the culture medium prior to inoculation, and of whole cultures prior to removal of leptospirae were employed as control antigens.

Serological demonstration of antigens was made by the quantitative complement fixation technic of Kolmer(8). Hyperimmune serums against each leptospiral strain studied were prepared in rabbits. An immunization schedule of 4 intravenous injections of living leptospirae at weekly intervals was employed. The initial dose was 0.5 ml of culture, followed by 1.0 ml, 2.0 ml, and 4.0 ml doses. Rabbits were bled on the fifth day after the last injection. Serums were separated from the clotted blood, filtered through Seitz E-K pads and stored at –20°C until used.

TABLE I. Demonstration of Leptospiral Soluble Antigen by Quantitative Complement Fixation.

Antigen	Dilution of antiserum, 1: 10	20	40	80	160	320	640
Uninoculated culture medium	—	—	—	—	—	—	—
Whole culture	4+	4+	4+	4+	4+	3+	—
Leptospirae-free culture liquor	4+	4+	4+	4+	4+	3+	—
Sonic-vibrated* leptospiral suspension	4+	4+	1+	—	—	—	—

* Diluted to equivalent of original culture volume.

Results. Comparative quantitative complement-fixing antigen titrations of whole leptospiral culture, leptospirae-free culture liquor, sonic-vibrated leptospiral suspension, and uninoculated culture medium are shown in Table I. This titration clearly demonstrates the presence of a soluble complement-fixing antigen in the organism-free culture liquor. Similar antigens were likewise demonstrated in the leptospirae-free culture liquor of all other leptospiral strains studied.

Quantitative complement fixation studies with the soluble antigen on hyperimmune serums prepared against homologous and heterologous leptospiral strains, indicate that this antigen-antibody reaction is highly strain specific when critical antigen dilutions are employed. Table II illustrates the effect of

TABLE II. Effect of Antigen Concentration upon Strain Specificity.

	L. canicola soluble antigen									
	Diluted 1:2					Diluted 1:4				
	Serum dilution, 1:					Serum dilution, 1:				
Hyperimmune serum against	4	8	32	128	512	4	8	32	128	512
L. pomona	+	+	—	—	—	—	—	—	—	—
djasiman	+	+	—	—	—	—	—	—	—	—
sentot	+	+	—	—	—	—	—	—	—	—
autumnalis	+	+	—	—	—	—	—	—	—	—
ballico	+	+	—	—	—	—	—	—	—	—
ballum	+	+	—	—	—	—	—	—	—	—
canicola	+	+	+	+	+	+	+	+	+	+
icterohemorrhagiae	+	+	—	—	—	—	—	—	—	—
grippotyphosa	+	—	—	—	—	—	—	—	—	—
medanensis	+	+	—	—	—	—	—	—	—	—

TABLE III.
Quantitative Cross Complement Fixation.

Antiserum	Soluble Antigen 1	2	3	4	5	6	7	8	9	10	11	12
1.	$\boxed{T}$	$\frac{T}{4}$	$\frac{T}{16}$	$\frac{T}{64}$	$\frac{T}{128}$	$\frac{T}{128}$						
2.		$\boxed{T}$	$\frac{T}{32}$	$\frac{T}{16}$	$\frac{T}{16}$	$\frac{T}{128}$	$\frac{T}{16}$					
3.			$\boxed{T}$	$\frac{T}{64}$		$\frac{T}{64}$						
4.		$\frac{T}{4}$	$\frac{T}{4}$	$\boxed{T}$	$\frac{T}{4}$	$\frac{T}{128}$						
5.		$\frac{T}{8}$	$\frac{T}{32}$	$\frac{T}{16}$	$\boxed{T}$							
6.		$\frac{T}{8}$	$\frac{T}{16}$	$\frac{T}{64}$	$\frac{T}{128}$	$\boxed{T}$	$\frac{T}{16}$					
7.		$\frac{T}{8}$				$\frac{T}{64}$	$\boxed{T}$					
8.		$\boxed{T}$	$\frac{T}{16}$		$\frac{T}{16}$			$\boxed{T}$	$\frac{T}{32}$			
9.		$\frac{T}{8}$		$\frac{T}{128}$	$\frac{T}{128}$		$\frac{T}{32}$		$\boxed{T}$			
10.							$\frac{T}{16}$			$\boxed{T}$		
11.									$\frac{T}{32}$	$\frac{T}{32}$	$\boxed{T}$	
12.											$\frac{T}{64}$	$\boxed{T}$

T= Titer obtained with homologous antiserum

Leptospiral strains:

1. *L. pomona*
2. *L. autumnalis,* Akiyami A
3. *L. icterohemorrhagiae,* Wijnberg
4. *L. djasiman*
5. *L. sentot*
6. *L. canicola*
7. *L. ballum*
8. *L. autumnalis,* Fort Bragg
9. *L. grippotyphosa,* Moscau V
10. *L. pyrogenes,* alexi
11. *L. andaman*
12. *L. semerang*

antigen dilution upon strain specificity.

The strain specificity of these antigens was further evidenced in cross complement fixation studies. Results of these examinations are shown in Table III.

Concentration, purification, and characterization of these antigens is currently in progress. Preliminary results obtained in this laboratory(9) indicate that the antigens are alcohol soluble materials which withstand heating at 100°C for 30 minutes with no appreciable loss of serological reactivity. The materials fail to illustrate biuret or ninhydrin reactions, but react positively in the Molisch test.

Discussion. Previously prepared leptospiral complement-fixing antigens have been prepared from the organisms or from extracts of washed organisms. One may reasonably infer from their method of preparation, and from their broad antigenic spectra, that they are somatic, and neither surface nor soluble antigens. The substances herein described are clearly soluble antigens exhibiting a high degree of strain specificity.

The use of complement-fixing antigens in serological studies on the leptospiroses has, in the past, been limited by the method of preparation of the antigens. The slow growth rate and low maximum population density of leptospirae in culture, as well as the expensive mediums required for their propagation, makes preparation from the organisms laborious and costly. In addition, the limited availability of equipment such as sonic vibrators and freeze-drying apparatus has limited the number of laboratories capable of preparing such antigens. In marked contrast, is the ease and low cost of preparation of the soluble antigens. Yager and his associates(10) have reported studies demonstrating the many advantages of the use of sonic-vibrated complement-fixing antigens as diagnostic agents for acute leptospirosis *per se.* However, as Collier and Mochtar(11) and Gsell(12) point out, identification of the infecting strain may often be desirable. It appears, that in these instances, the specific soluble complement-fixing antigens might advantageously supplement the broader screening antigens.

Summary. A soluble, specific complement-fixing antigen, or antigen complex, was demonstrated in the organism-free culture liquor of each leptospiral strain studied. The antigenic material is heat stable and nonprotein in nature. The antigens are present in amounts which make feasible their employment in serological investigations of the leptospiroses.

The authors wish to express their thanks to the staff of the Department of Serology for furnishing standardized complement, amboceptor and erythrocyte suspensions.

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Presence of a Leukotaxine-like Substance in Extract of Severely Injured Muscles. (19577)

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Recently(1) the writer expressed the view that leukotaxine, the substance primarily responsible for the increased capillary permeability and the migration of leukocytes in inflammation, is formed whenever cells are severely injured; consequently one cannot really speak any longer of "normal tissue" as is done by Moon and Tershakovec(2,3) when one macerates tissues and prepares an extract from them. One would expect to recover either leukotaxine or a substance closely resembling leukotaxine in such extracts of severely injured cells. In 1937, (unpublished data) the writer has obtained such a leukotaxine-like substance from beef heart extract. The reason for not continuing and publishing these studies was neither to go on a tangent nor to reason by analogy, but only to center on his main theme which was inflammation and its natural product, namely the exudate. As stated in his discussion(1) leukotaxine and its polypeptide nature, has been frequently confirmed.

In the present communication, observations are reported on the presence of a leukotaxine-like substance in the saline extracts obtained from muscles of rabbits as a result of grinding such tissues in a mortar. Such injured cells yield essentially the same factors as obtained from inflammatory exudates(4,5).

Method. Rabbits were killed by injecting air intravenously. The muscles of the thigh were immediately removed and weighed. The tissue was cut up in small fragments and treated with physiological saline. The amount of saline utilized was approximately equal to a volume in cubic centimeters of twice the weight of the muscle tissue as measured in grams. Some of this whole saline extract was tested on the skin of normal rabbits by the intracutaneous injection of 0.5 cc of the material on the abdominal surface. Five to 6 cc of 1% trypan blue in saline was then injected intravenously. Readings on the accumulation of the dye in the treated area were recorded periodically for about 1 to 2 hours. The rest of the saline preparation was extracted for the presence of a leukotaxine-like substance by only slightly modifying the method described previously for the extraction of leukotaxine from inflammatory exudates (6). The scheme of extraction utilized may be stated as follows: the saline extract of the thigh muscles was treated with dioxan (1:1) and stirred for about two hours. The mixture was then centrifuged and the supernatant phase treated with an equal volume of acetone. A precipitate resulted. This was centrifuged off and the supernatant evaporated to dryness *in vacuo* at ±50°C. To the dried material an amount of butyl alcohol equal to ½ the volume of the acetone supernatant-treated fraction was added, and the mixture was stirred for about one hour. This was then concentrated *in vacuo* at ±55°C to about 1/10th volume. At that point the butyl alcohol concentrate was divided into two parts. One part contained a granular-like precipitate

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* Also Fellow of the John Simon Guggenheim Memorial Foundation.

TABLE I. Effect of a Leukotaxine-Like Substance from Muscle Extract on Capillary Permeability and on the Migration of Leukocytes.

Rabbit No.	Leukotaxine-like material inj. in skin	ACTH intrav., mg	Duration of inflamma-tion, hr:min	Accumulation of trypan blue in the treated areas (perme-ability test)	Diapedesis of leukocytes in treated area (chemotactic response)
43–57	C		0:44	2+ to 3+	+ to 2+
–81	C–D		0:57	3+	+
–70	C		1:28	4+	2+
–48	C		1:30	4+	2+
–83	C fraction		2:10	3+	3+
–80	C–D	20	0:57	4+	0
–80	Same	20	1:07	4+	
–69	C	30	1:25	2+*	
–82	C	20	2:09	3+	Faint trace
–97	Whole saline extr. of muscle		1:04	3+	2+
–98			1:15	+ to 2+	3+
–96			1:29	Trace	2+
–94			2:24	+	3+
–95		15	1:26	0	0
–93		15	2:25	0	3+

* At periphery.

which was centrifuged and the resulting precipitate dried in a desiccator. This was the so-called C-fraction. The other part of the butyl alcohol concentrate was evaporated to dryness, yielding a viscous and perhaps slightly tarry material. This was the so-called C-D-fraction.

These two fractions were suspended in 0.5 cc of saline and injected intracutaneously into the skin of the abdomen of rabbits. This was followed, as stated above, by the intravenous injection of trypan blue and readings on the accumulation of dye were accordingly made. At the termination of the observations, the skin areas were excised and fixed in 10% formaldehyde for subsequent microscopic examination.

Results and discussions. The results obtained are assembled in Table I. It is clear that the C-fraction or C-D-fraction of leukotaxine-like material, obtained in a manner similar to leukotaxine from exudative material, induce both an increase in capillary permeability and the migration of leukocytes into the treated skin area (Table I, Fig. 1.) Furthermore, it has been shown that ACTH does not suppress the increase in capillary permeability to trypan blue caused by leukotaxine(7), while at times it inhibits the diapedesis of leukocytes especially when the leukotaxine preparation is not too potent(7). The same finding has been observed with the leukotaxine-like fractions from muscle tissue extract following severe injury. This would support the contention that the fractions obtained are essentially not different from leukotaxine, as obtained from inflammatory exudates. Finally, these fractions likewise fail to contract the isolated segment of guinea pig intestine. This is precisely similar to the response of the intestine to leukotaxine from exudates(6). The only point of difference is the negative Biuret and ninhydrin tests which are found, however, to be positive in the case of leukotaxine(6). The amino nitrogen before and after hydrolysis suggests that perhaps one is dealing with a dipeptide. The nature of the prosthetic group, if present, is probably different in various tissues and from that in the leukotaxine of inflammatory exudates. The total nitrogen by Pregl's micro-Kjeldahl method is found in six determinations to average 5.52%. The whole saline extract tends to induce likewise an increase in capillary permeability and local migration of leukocytes. When ACTH is added, however, both the increase in capillary permeability and the migration of leukocytes in one instance seemed to be

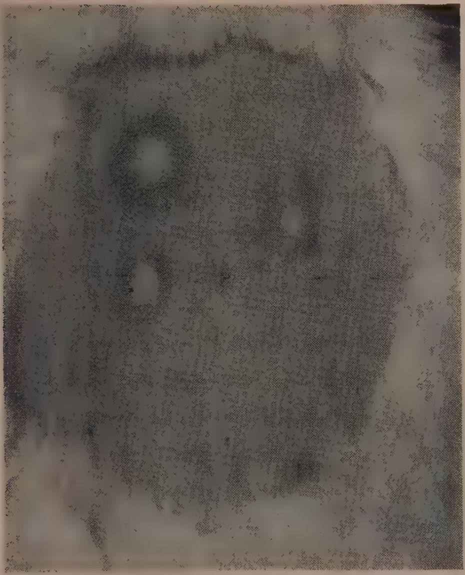

FIG. 1. Rabbit 4380. 20 mg of ACTH in 2 cc of saline inj. intrav. In upper left area .5 cc of the C–D–fraction from muscle saline extract inj. intracut. In lower left area .4 cc of the C–fraction has been inj. On the right side .4 cc of the C–D–fraction has been introduced, while physiological saline has been inj. intracut. in lower right side. 6 cc of 1% trypan blue in saline was then intrav. inj. About 40 min later present photograph taken. Note that intrav. inj. of ACTH failed to suppress increase in capillary permeability to trypan blue caused by the leukotaxine-like fractions C–D and C of muscle extract.

suppressed (Table I). The reason for this is not clear. It is possible that in the whole extract there is also an abundance of exudin which is in turn suppressed by ACTH(7); *i.e.*, as far as increased capillary permeability is concerned.

Moon in reply to the writer's comments(1) failed to answer the fundamental criticism, namely: do normal tissues contain leukotaxine? The writer carefully pointed out(1) that only injured cells, such as found in inflammation or by macerating tissues have their biochemistry so altered as to produce various common denominators, such as leukotaxine, which in turn are capable of accounting for some of the biological aspects of the very complex problem of inflammation. This does not mean that the writer believes that leukotaxine is present in all normal tissues, but rather it is his belief that it can only be formed during injury of cells. The present paper supplies further factual information toward the problem. Tershakovec and Moon (8) simply showed that numerous substances as well as protein split products increase capillary permeability and induce leukocytic migration. In the discussion, Moon questioned whether the present writer would infer that leukotaxine is present in all these substances. The answer is quite clear,(6,9). All these substances are essentially inflammatory irritants which injure the cells of the host. These cells, in turn, have their biochemistry altered by injury so that leukotaxine is formed which accounts for the standard reaction of local increased capillary permeability and leukocytic migration. It is also conceivable that perhaps some of the protein split products utilized contain, in addition, some leukotaxine-like material (10, 2nd reference), which thus accentuates the full permeability and chemotactic effect, but this possibility is not essential in order to elicit some degree of increased capillary permeability and chemotactic response by directly injuring the cells of the host.

As pointed out by the writer(10) when discussing the two factors in leukotaxine: "The analytical procedure has thus failed to dissociate the two factors. Although this type of evidence is strongly suggestive of a single substance, it is, however, to be borne in mind that only the certitude of complete chemical purity of the material can establish this as a definite fact." Thus far, although there may be two separate substances in leukotaxine, such a dissociation in exudates has not been accomplished. Spector has recently succeeded to do this in the digest of fibrin; but he has not been able to report any such dissociation in inflammatory exudates(11).

Finally, in regard to the criticism concerning the multiplicity of factors(1), this point has been discussed elsewhere(9,12). It simply reflects the complexity of the problem of inflammation. The analytical procedure employed has yielded in inflammatory exudates separate specific factors which are responsible for only specific biological processes in inflammation. These factors have been separated chemically and identified biologically. The future will yield, as in most biological entities, greater chemical accuracy in their identification. Thus far, pyrexin and the thermostable leukocytosis factor have been brought to the state of ready crystallization.[†] Many of the other factors have been identified as polypeptides. Their chemical structures, however, have yet to be identified by chemists.

Conclusions. Severely injuring muscle tissue of a rabbit by mechanical means induces the formation of a leukotaxine-like substance which has essentially the biological attributes of leukotaxine as obtained from inflammatory exudates. The fractions of leukotaxine-like material induce an increase in capillary permeability and cause leukocytic migration. The isolated guinea pig intestine fails to contract when treated with these fractions, while histamine added subsequently contracts the segment. This would indicate that the leukotaxine-like fractions obtained do not appear to be histamine.

[†] Studies now in progress indicate the possibility that the thermostable leukocytosis factor may be adsorbed on a non-specific crystalline structure, but this will be taken up *in extenso* in a subsequent communication.

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Efficacy of 1-Ethanesulfonyl-4-Ethyl-Piperazine Hydrochloride in Treatment of Controlled Hemorrhagic Shock.* (19578)

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Acute hemorrhage is of frequent occurrence from industrial and highway accidents and battle casualties. While its treatment has been facilitated greatly by the ready availability of supplies of plasma and whole blood, the time required to obtain them and for the proper matching of blood still may cause such a delay that successful reversal of the pathologic state is not produced quickly enough and death ensues. The development of a drug which would be satisfactory in delaying the onset of an irreversible state consequently is highly desirable. In preliminary studies, Bovet *et al.* reported that 1-ethanesulfonyl-4-ethyl-piperazine hydrochloride (R.P. 3885) apparently is successful in protecting rats from traumatic shock produced by the Noble-Collip drum(1). Later, these workers indicated that this compound was successful in increasing the survival time of dogs subjected to "traumatic-hemorrhagic"

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[*] Aided by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

[†] International Educational Exchange Program of The Department of State, from Severance Union Medical College, Seoul, Korea.

[‡] The drug was synthesized and generously supplied to us as a pure crystalline compound by Dr. Thomas P. Carney, The Eli Lilly Co., and in a 25% solution, which was slightly amber in color, by Dr. Jacob W. Stutzman, of Smith, Kline & French, Inc.

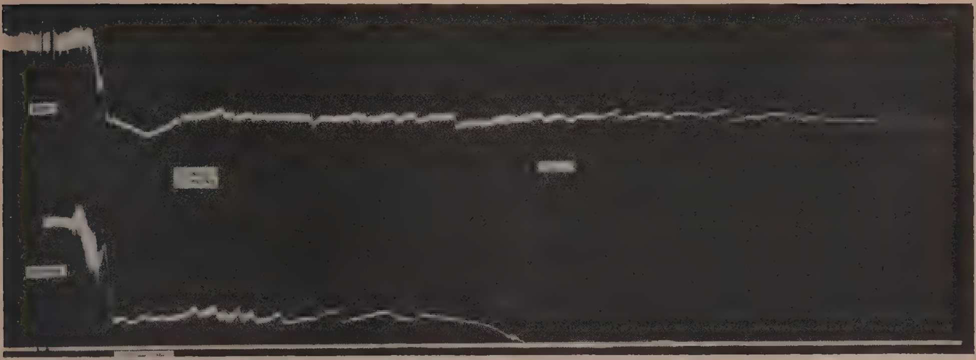

FIG. 1. Kymographic records of blood pressure from one of the paired experiments in which duration of survival was determined after production of hypotension. In the tracing indicated by "DRUG," 50 mg/kg of piperazine compound was administered intrav. in 10 min. Control animal survived for 3 hr, the treated animal 4½ hr.

shock(2). Others have reported that the drug was ineffective in the treatment of traumatic shock produced in rats(3).

The present report deals with a preliminary survey of the efficacy of 1-ethanesulfonyl-4-ethyl-piperazine hydrochloride‡ in two types of experiments, in which acute hypotension was produced by controlled hemorrhage in dogs. In the first series of experiments the time of survival was determined in paired dogs, one of which served as the control while the other was treated with the drug. In the second type of experiment similar procedures were followed, but the blood of each dog was reinfused and its recovery was sought.

Method. Healthy mongrel dogs maintained on a standard *ad libitum* diet of dried dog biscuits were paired as nearly as possible as to sex, age, general breed, and weight. Unconsciousness was produced with 30 mg of pentobarbital sodium per kg, given intravenously in about 3 minutes. Endotracheal intubation was employed in most of the dogs so that a free airway was maintained at all times. Two mg heparin per kg also was given intravenously to aid in preventing clotting while arterial blood pressure was determined from the femoral artery with a mercury manometer and recorded graphically. The opposite femoral artery was cannulated for bleeding and connected to a receiving flask containing a preservative solution consisting of 1.38% sodium citrate, 1.58% dextrose, and 0.5% citric acid. The hypotensive state was produced essentially in one stage, a modification of the technic of Wiggers(4), by permitting blood to flow rapidly and simultaneously into an individual container for each dog of the pair until a hypotension of 40 mm Hg was attained within approximately 5 minutes. Small volumes of blood were withdrawn subsequently to maintain the initial hypotensive level. The first of the 2 general types of experiments was performed on 13 pairs of dogs. After blood was withdrawn to produce the hypotension, one animal of the pair was treated intravenously with 50 to 100 mg of the drug per kg while the other served as the control. The time of survival then was determined for each animal. In a second series of 17 pairs of dogs, hypotension again was produced and one animal of each pair was treated within the first 30 minutes or after 90 minutes. Some 150 to 180 minutes after the onset of hypotension, the withdrawn blood was reinfused into the respective treated and control animals and their recovery was determined. During the course of the experiments specific gravity was measured by the copper sulfate method. Postmortem examinations were performed on most of the animals which succumbed.

Results. A record of an experiment in which length of survival was tested is given in Fig. 1. The drug was injected intravenously after the first 10 minutes of hypotension and the blood pressure was maintained at a level that did not exceed 40 mm Hg. At the end of

TABLE I. 7 experiments Nov. 17 to Dec. 21, 1951 in which, after production of a hypotension of approximately 40 mm Hg, length of survival of dogs was determined. 50 mg/kg piperazine was administered within 30 min of production of hypotension. No transfusion was given these animals.

	Control animals Survival time, hr	Control animals Survival time, min	Treated animals Survival time, hr	Treated animals Survival time, min
	1	45	4	25
	2	56	2	53
	2	44	2	5
	2	50	4	28
	3	25	8	41
	2	45	1	10*
	1	53	4	20
Avg	2	37	4	0

* Toxic reaction to drug.
t = 1.5 with 12 degrees of freedom.

TABLE II. 6 Experiments of Jan. 2-14, 1952 in Which Duration of Survival Was Determined When Piperazine Was Given 90 Min after Production of the Hypotensive State. No blood was administered to this group of animals.

	Control animals Survival time, hr	Control animals Survival time, min	Dose, mg/kg	Treated animals Survival time, hr	Treated animals Survival time, min
	5	12	50	8	6
	2	50	60	2	25
	2	52	50	2	30
	3	0	50	3	55
	3	50	50	2	35
	2	55	50	2	45
Avg time of survival	3	24		3	42

3 hours the control animal died, whereas the treated dog lived for 4 hours and 30 minutes.

The data from a series of 7 pairs of animals in which the drug was injected within 30 minutes after the production of hypotension are given in Table I. The survival time was measured from the beginning of hypotension until death. In Exp. 6 the treated animal died in the extraordinarily short time of one hour and 10 minutes. Since there was no instance for any of the control animals of such a brief time of survival, it seems quite possible that toxicity of the drug was responsible. The average survival time for the treated animals was 4 hours and for the control group 2.6 hours. Statistical analysis of these figures reveals that the difference in survival time is significant since, by the Fischer formula, t = 1.5, for 12 degrees of freedom.

In a series of 6 pairs of animals the drug was administered after 90 minutes of hypotension. The average survival time for the two groups was essentially the same, since that for the treated animals was 3.7 hours while for the controls it was 3.4 hours. The data are given in Table II and indicate that when treatment is delayed until this late in the hypotensive period, survival time is not prolonged.

A graphic record of the blood pressure from an experiment in which there was reinfusion of blood is given in Fig. 2. Intravenous injection of the piperazine was begun within the first 10 minutes of hypotension and 10 to 15 minutes was taken for its infusion. The blood pressure level in the treated animal subsequently stabilized at 20 mm Hg. This effect

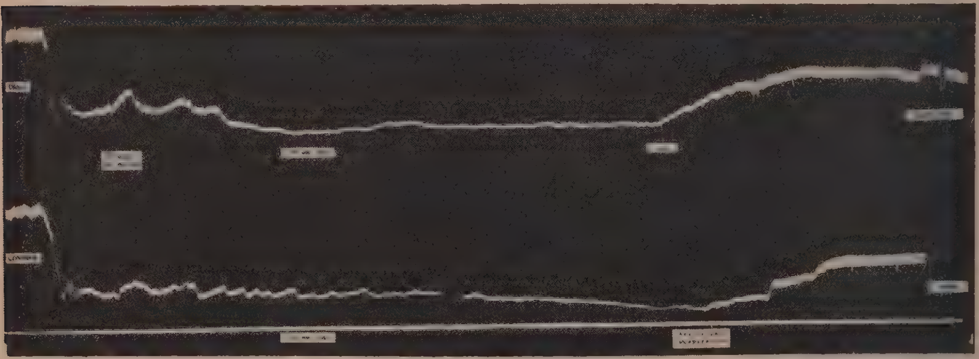

FIG. 2. A record from paired experiments in which administration of 50 mg/kg of the piperazine drug to one of the dogs was started 10 min after hypotension was established by acute hemorrhage. Blood was reinfused into each animal after 3 hr. Control animal died 8 hr later but the treated (drug) animal survived.

TABLE III. 12 Experiments Conducted Between Jan. 16 and Feb. 18, 1952 in Which There Was Reinfusion into Each Animal of Each Pair of the Blood Withdrawn. Administration of the drug was made intravenously within 30 min of production of the hypotension.

Time of reinfusion for each dog, hr	min	Control animal Initial mean B.P., mm Hg	Control animal Result	Piperazine treated animal Initial mean B.P., mm Hg	Piperazine treated animal Dose, mg/kg	Piperazine treated animal Result
1	25	180	D*	160	50	S*
3		155	D	145	50	S
2	40	150	S	140	50	S
2	5	150	D	120	75	S
2	50	160	D	140	100	S
2	20	140	D	130	100	S
2	50	170	D	130	100	S
2	35	130	D	120	100	S
3		140	D	130	100	S
1	20	120	D	140	100	D
3		135	D	140	100	S
3		160	S	140	100	S
Survival†			17%			92%

* D = died; S = survived.
† Statistical analysis by the "t" test reveals p = .0003.

TABLE IV. 5 Experiments Performed Feb. 20-29, 1952 in Which the Blood Withdrawn to Produce Hypotension Was Reinfused into the Pairs of Animals at the Times Indicated. Administration of the piperazine drug was made at 90 min.

Time of reinfusion for each dog, hr	min	Control animal Initial mean B.P., mm Hg	Control animal Result	Piperazine treated animal Initial mean B.P., mm Hg	Piperazine treated animal Dose, mg/kg	Piperazine treated animal Result
3	10	145	D*	150	75	D
2	28	165	D	150		
2	30	140	S	130		
2	35	140	S	140		
2	32	140	D	140		

* D = died; S = survived.

occurred shortly after injection of the drug and indicates that the piperazine compound may impose an added increment on the hypotension resulting from hemorrhage. The slow rate employed for its administration was an attempt to minimize this action. During the same period the blood pressure of the control animal was maintained at 40 mm Hg. At the end of 3 hours of hypotension, each animal had its withdrawn blood reinfused. The control animal was deemed to be in a very critical condition at this time and artificial respiration was administered during the first 30 minutes of reinfusion. Both animals regained a blood pressure level which was somewhat lower than their initial normal levels. The treated animal survived while the control one died 8 hours after the reinfusion of its blood.

The results from a series of 12 pairs of animals in which the drug was injected within the first 30 minutes of hypotension are given in Table III. The withdrawn blood was reinfused after 80 to 180 minutes of hypotension. These results are considered to be highly significant since statistical analysis by the "t" test reveals p = .0003.

Data from a series of 5 pairs of animals in which administration of the piperazine was withheld until there had been 90 minutes of hypotension are given in Table IV. Reinfusion of the withdrawn blood was instituted at 2½ to 3 hours. The mortality of 100% in the treated animals indicates that, after such a prolonged hypotensive period, no benefit obtains from administration of the drug. Two of the control animals of this group obviously had not reached an irreversible state since they survived the hypotensive period.

Postmortem examinations revealed the usual morphologic changes characteristic of hemorrhagic shock. Changes in specific gravity likewise followed the characteristic pattern previously reported.

Discussion. Rigidly controlled experiments suggest that within definite limits 1-ethanesulfonyl-4-ethyl-piperazine administered intravenously is effective in prolonging the survival time and decreasing the mortality in dogs subjected to extreme hemorrhagic shock. The protective action of the drug occurred when it was administered in a dosage of 50 to 100 mg/kg within the first 30 minutes of hypotension. No protection was evident when hypotension was present for 90 minutes before the drug was injected.

The procedures used varied somewhat from others previously employed in testing a drug for the treatment of hemorrhagic shock(5). Preliminary experiments suggested that a 1½ to 2 hour period of hypotension did not consistently produce a state of irreversible shock. The period of hypotension was therefore prolonged to as much as 3 hours with an average of 2 hours and 30 minutes. To obviate variations of seasons of the year, weather, and technic, all experiments were done on paired dogs, so that a control was run simultaneously with the experimental animal.

The pharmacodynamic actions and toxicity of 1-ethanesulfonyl-4-ethyl-piperazine were not determined in these experiments although it was determined that larger doses could be injected much more rapidly in normotensive dogs. Thus, a definitely increased toxicity was present in some of the hypotensive animals. Evidence of this was the pronounced drop in blood pressure which resulted soon after administration of the drug for which compensation did not occur for approximately 30 minutes (Fig. 2). In several animals compensation never occurred and death ensued rapidly (Dog 6, Table I). During injection of the drug a rather marked increase in respiratory rate and depth usually was evident.

Summary. 1. When dogs which had been subjected to severe hemorrhage were treated within 30 minutes with 50 to 100 mg/kg of 1-ethanesulfonyl-4-ethyl-piperazine administered intravenously, survival time was prolonged or mortality decreased in comparison to untreated control animals. 2. No protective action was evident when treatment was withheld until there had been 90 minutes of hypotension. 3. The piperazine compound is definitely toxic and was considered responsible for death of 3 of 30 dogs in which it was used for treatment. 4. The drug imposed a decided hypotensive factor on the acute 40 mm Hg state produced deliberately by controlled hemorrhage.

Dr. William Young, Chief of Surgery at the Veterans Administration Hospital, Madison, helped institute work on this project.

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Platelets: VII. Shortened "Platelet Survival Time" and Development of Platelet Agglutinins Following Multiple Platelet Transfusions.* (19579)

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In the course of studies on the fate of platelets injected into patients with various types of thrombocytopenia(1,2), it was observed that the survival time became shorter or, more strictly, the rate of disappearance of platelets from the circulation was accelerated when the patients received repeated transfusions of platelet-rich polycythemic blood or suspensions of isolated platelets. At the same time, it was discovered that platelet agglutinins developed occasionally.

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* Work performed under contract with Atomic Energy Commission.

† Damon Runyon Senior Clinical Research Fellow.

‡ Damon Runyon Clinical Research Fellow.

Materials and methods. Because of various abnormal and possibly immuno-humoral factors in idiopathic thrombocytopenic purpura (2-5), patients with this disease were excluded from the present series. Serial determinations of platelet survival time were conducted in 4 cases, 2 with hypoplastic and 2 with aplastic anemia. These patients presented severe thrombocytopenia requiring repeated transfusions of platelet-rich polycythemic blood or of isolated platelets for the control of bleeding. Simultaneously, the serum was studied for platelet agglutinating properties in these 4 patients and in 11 additional patients who had received multiple transfusions of blood over a period of months.

(a) *Technic for the determination of survival time of injected platelets.* Transfusions of platelet-rich polycythemic blood or plasma were given by the direct technic previously described(2). The blood or plasma administered was of homologous group and type and compatible as determined by cross-matching in albumin medium. Moreover, blood was given only after a mixture of inactivated, decalcified and deprothrombinized serum of the recipient and saline suspension of platelets from the donor had failed to show clumping after 12 hours of incubation at room temperature in Silicone-coated tubes, thus presumably indicating the compatibility of platelets. Clot retraction, bleeding time, capillary fragility and utilization of prothrombin during the coagulation of blood were followed in each patient with the procedures previously described(2). Platelet count was determined by both the indirect method of Dameshek(6) and by a direct method(7). Values were considered significant only if in relative agreement. The figures shown are those obtained with the indirect method.

(b) *Detection of platelet agglutinins in serum.* 1) *Preparation of platelet suspensions.* Platelet suspensions were freshly prepared and all procedures were carried out in Silicone-coated glassware. After their separation from plasma components and the various cellular elements of the blood, platelets are reasonably well preserved even when in contact with foreign surfaces such as glass. Nevertheless, the use of non-wettable surfaces decreases the loss or irreversible agglutination of platelets during their separation. We have also noticed that when ordinary glassware is used human platelets may at times become less agglutinable by specific anti-human platelet sera obtained from animals. Blood was collected from a donor's vein by the 2-syringe technic(8), through Silicone-coated needles in Silicone-coated syringes; it was then transferred to 15 ml Silicone-coated test tubes containing 1/10 volume of 0.2 M sodium citrate. More recently, 1% Sequestrene-Na_2§ in 0.7% sodium chloride solution has been used routinely. The blood was spun in a refrigerated centrifuge at 500 r.p.m. for 15 minutes, the plasma separated and allowed to stand for one hour at room temperature, and finally centrifuged for 10 minutes at 1,000 r.p.m. to separate most of the remaining red and white cells.‖ The platelets were then collected at the bottom of the tube by centrifuging at 2,000 r.p.m. for 20 minutes, and resuspended in a small volume of saline solution containing 0.2% sodium acetate. The platelet suspension was then diluted with saline-acetate solution to obtain a final count of approximately 500,000 platelets/cu mm by a direct method(7). The suspension was considered satisfactory only when free of clumping on microscopic reading and containing less than one red or white cell per high power microscopic field. 2) *Preparation of serum for agglutination studies.* Blood was collected from an antecubital vein, allowed to clot at 37°C for one hour, and the serum separated by centrifugation. After incubation at 56°C for 30 minutes to inactivate complement, serum was divided in small aliquots and these kept frozen at —20°C until used (20 days to 4 months). Since we have previously shown that addition of platelets to serum containing prothrombin, calcium and other coagulation factors will result in non-specific agglutination of platelets, the serum was decalcified and/or deprothrombinized by the technics previously described(10). Dilutions of serum were prepared with phosphate-saline buffer (pH 7). 3) *Agglutination test.* The blood type and group of the patient under investigation were first determined and platelets from a compatible donor prepared. 0.1 ml of platelet suspension and 0.1 ml of serum at various dilutions were transferred to 8 x 50 mm serology tubes coated with Silicone by means of Silicone-coated pipettes, the mixture allowed to stand at room temperature for one hour and kept overnight at 4°C. Alternatively, plasma rich in platelets, as obtained by low speed centrifugation (10 minutes at 500 r.p.m.) of blood collected in Sequestrene-Na_2, could be used, provided the serum had been inactivated, decalcified and/or deprothrombinized and the platelet count adjusted to 500,000/cu mm diluting, if necessary, with platelet-poor plasma obtained by prolonged centrifugation at high speed. Two control test tubes were also prepared: one containing 0.1 ml of buffered saline and 0.1 ml of platelet suspension and the other a mixture of 0.1 ml of platelet suspension and 0.1 ml of normal human serum kept frozen at —20°C for 20 days and inactivated, decalcified and/or deprothrombinized prior to use. After overnight incubation, all test tubes were returned to room temperature for 30 minutes and the platelets (which had settled to the bottom) resuspended by gently tapping the bottom of the tube. More vigorous tapping of the tube, although reducing the size of the clumps, failed, in all cases, to cause complete resuspension of the agglutinated platelets. Presence of clumping was read macroscopically, over a concave lens giving a X3 enlargement under a strong light, and microscopically, under high dry objective. Small drops of Silicone were observed in the field at times, but they did not interfere with the reading. All experiments were run in duplicate and, in order to minimize the human error of the technic, the results were read by the same two observers, and only the results in agreement accepted as valid. The procedure described has been currently employed during the past year for the search of antiplatelet substances in serum of patients with idiopathic thrombocytopenic purpura and secondary thrombocytopenia. A series of 112 normal sera has been completed thus far and negative results have been consistently observed provided the serum has been kept frozen at —20°C for 20 days and inactivated, decalcified and/or deprothrombinized prior to use. The mechanism of the ability of fresh normal sera to agglutinate platelets will be discussed in another communication(11). By this method, also, platelet agglutinin have been demonstrated in a number of cases of isopathic thrombocytopenic purpura.

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§ Disodium ethylenediaminotetraacetate dihydrate, obtainable from the Akrose Chemical Co., Providence.

‖ Addition of 1/10 volume of 2% sol. of Tween-80 (Rohm and Haas, Philadelphia) to plasma prior to centrifugation allows resuspension of platelets in saline with minimum loss due to clumping. Following Minor and Burnett(9) we have now substituted Triton W-1339 for Tween-80 with even more satisfactory results and use this procedure routinely. The minute amount of the surface active agent which remains with platelets does not appear to influence their agglutinability nor cause spontaneous clumping. A Sequestrene-Triton technic, to be described, is now used routinely in this Laboratory for the preparation of large number of platelets for use in humans.

Results. a) *Survival time of injected platelets after repeated platelet transfusions.* The 4 patients in whom the rate of disappearance of platelets injected by direct transfusion of platelet-rich polycythemic blood was studied at intervals of time had received frequent transfusions of fresh blood in plastic bags, isolated platelets and polycythemic blood by direct transfusion(2). In 3 of these patients the survival time of injected platelets became progressively shorter and, simultaneously, the beneficial effect of platelet transfusions on the bleeding manifestations, bleeding time, capillary fragility and coagulation process became less pronounced. A typical example is shown in Fig. 1. The platelet survival time of this patient, a case of hypoplastic anemia, was of approximately 4 to 5 days, on 5/12/1950. On 5/29/1950 he received a transfusion of 350 billions platelets suspended in plasma and on 6/13/1950 the platelet survival time had decreased to 3 days. Two transfusions of fresh blood collected by gravity and admin-

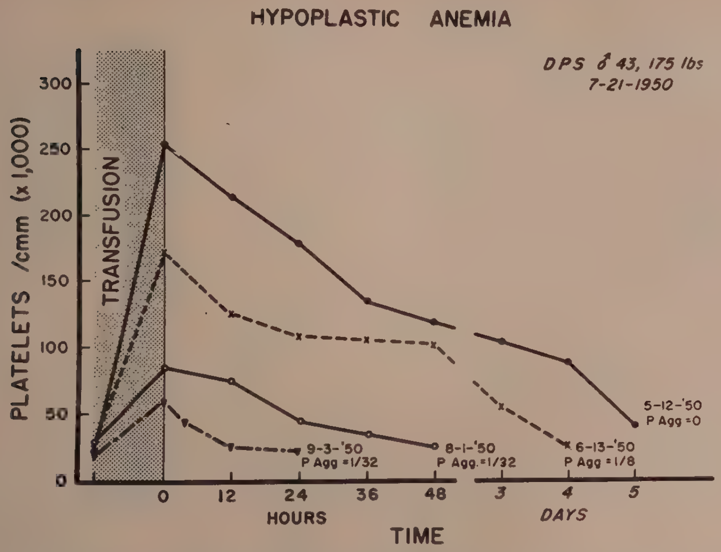

FIG. 1. Shortened survival time of injected platelets and development of a platelet agglutinin in serum of one patient receiving multiple platelet transfusions. (Shaded area represents time necessary to complete the platelet transfusions: 15 to 45 min).

istered with Silicone-coated bottles, connecting tubes and needles were given on 6/27/1950 and 7/12/1950. On 8/1/1950 the platelet survival time was reduced to approximately 24 hours and most thrombocytes had been quickly removed from the circulation. After another transfusion of 280 billions platelets resuspended in plasma on 8/24/1950, platelets injected on 9/3/1950 were very quickly removed from the circulation. At the same time, no improvement of petechial bleeding and epistaxes followed the last two platelet transfusions. The increased rate of utilization or destruction of platelets was also indicated by the extent of rise in the platelet level immediately after platelet transfusion which fell progressively shorter of the figure to be expected on the basis of the calculated number of thrombocytes injected and the patient's blood volume. b) *Development of acquired platelet agglutinins in patients receiving multiple platelet transfusions.* The serum of patient of Fig. 1 was found to have developed a platelet agglutinin, with a titer which rose progressively to 1/32, and remained until the patient's death from cerebral hemorrhage, being consistently demonstrable in several tests. The agglutinin did not seem capable of affecting the functional ability of normal platelets, as demonstrated by the following tests. Normal platelet-rich native human plasma was obtained by centrifugation at 500 r.p.m. at 4°C for 10 minutes of blood collected in Silicone. 0.3 and 0.5 of the patient's native serum were added to 0.7 and 0.5 ml of compatible native plasma respectively. In another series, normal native serum was added to normal native plasma in equal proportions. All mixtures were allowed to clot at 37°C and the retraction of the clot and the prothrombin activity of serum determined by methods previously described(8,12). No differences were observed between mixtures containing normal serum and those containing serum with the platelet agglutinating property.

Agglutinins against platelets were not found in the sera of the other 2 patients who had developed increased "resistance" to transfused platelets. In view of these findings, search for platelet agglutinins was conducted in the serum of 11 patients who had received multiple transfusions of blood within a few months. They included 2 cases with bleeding peptic ulcer, 2 cases with prostatic carcinoma and severe urinary bleeding, 3 cases of acute lymphocytic leukemia, 2 cases of Mediterranean anemia, and 2 cases of disseminated carcinomatosis with myelophtisic anemia. Six patients presented a normal or elevated platelet count, 4 a marked thrombocytopenia. One developed thrombocytopenia following multiple transfusions, as will be mentioned later. These patients received blood collected with the conventional technic in ACD solution and glass vacuum containers. This blood contained few platelets, as demonstrated by the result of direct count and its poor clot retraction on recalcification. Large amounts of platelet products were presumably present, however. since there was excellent utilization of prothrombin following recalcification and clotting of this blood.

A platelet agglutinin (titer ⅛), demonstrable consistently in tests taken at various intervals of time, was found in the serum of one of these patients, a case of bleeding peptic ulcer who had received 12 blood transfusions of fresh blood over a period of 45 days and whose serum, at the time of admission, had not shown platelet agglutinating activity. It dropped to a ¼ titer after 3 months during which the patient received no blood transfusions and was not detectable 6 months later. The platelet level of this patient had decreased from 850000/cu mm to 175000/cu mm and returned to values of 450000/cu mm following the disappearance of the platelet agglutinin in the patient's serum. The platelet survival time was found to be 2 days, as against the 4 to 6 days of most cases of secondary thrombocytopenia. The bone marrow did not present appreciable alterations in number or in activity of the megakaryocytes. Platelet count and platelet survival time returned to normal, following the disappearance of the agglutinin in the patient's serum.

Discussion. These observations indicate that, as patients with "secondary" or "amegakaryocytic" thrombocytopenia or even with normal platelet count are given repeated transfusions of platelets, the survival time of thrombocytes becomes progressively shortened and the beneficial influence on the bleeding manifestations less pronounced. After several platelet transfusions, the survival time of platelets may become as short as observed in idiopathic thrombocytopenic purpura, a condition in which the existence of platelet destructive mechanisms has been postulated (2-5). This finding suggests that mechanisms for platelet destruction have developed in some of these patients.

The pathogenetic mechanism involved is difficult to interpret, however. It should not be forgotten that changes in the course of the fundamental disease could be responsible for the acquired state of "resistance" to transfusion of platelets. The occasional discovery of platelet agglutinins in the serum of such patients suggests that, in particular cases, the development of "refractoriness" to the beneficial effect of platelet transfusions on the hemostatic mechanism might be due to iso-immunization against platelets or their products. Products of platelet disintegration must obviously possess antigenic properties, since the development of "refractoriness" to platelets and of a platelet agglutinin in a patient receiving blood with few intact thrombocytes was observed. The possibility cannot be excluded that iso-immunization might have been due to the repeated administration of incompatible platelets. Although we have collected some evidence for the existence of distinct platelet groups, observations of this type are handicapped by serious technical obstacles and require further careful evaluation. It is possible that these findings might be indicative of the mechanism through which thrombocytopenia develops in idiopathic thrombocytopenia purpura, or, at least, in some of these cases(13).

The cause of the decreased platelet survival in patients who had received multiple transfusions but failed to show any platelet agglutinin or platelet destroying agent in their serum remains obscure. At least part of these negative results could be due to the inadequacy of the technics presently available for the detection of anti-platelet agents.

Summary. 1. The rate of platelet disappearance or platelet survival time was followed at regular intervals in 4 patients with hypoplastic or aplastic anemia receiving repeated transfusions of platelet-rich polycythemic blood or of isolated platelets as supportive therapy. As transfusions were repeated, in 3 of these patients, a) survival time of platelets be-

came progressively shorter, b) administration of platelets was less and less effective in the control of the bleeding manifestations. In one patient a platelet agglutinin was detected in the serum. 2. Search for platelet agglutinins was conducted in the serum of 11 patients who had received multiple blood transfusions over a period of months. A low titer platelet agglutinin was detected in one case. This patient developed mild thrombocytopenia and exhibited shortened platelet survival time. 3. These findings suggest that "refractoriness" to the therapeutic effect of the administration of platelets may develop in patients receiving multiple platelet or blood transfusions. This may be due to the development of antiplatelet substances in the serum, at least in individual cases.

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Plasma Proteins in Venous and Cord Blood at Delivery Following Uncomplicated and Complicated Pregnancies. (19580)

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Protein metabolism via the human hemochorial placenta is still incompletely understood. The literature provides few determinations of the protein components in plasma from venous and cord blood samples of women at the end of uncomplicated and complicated pregnancies.

Preceding reports(1-5) have given electrophoretically determined plasma protein values for normal non-pregnant women, for women during and following uncomplicated pregnancy, and for women whose pregnancies were complicated by toxemias, diabetes, abruptio placentae, and hepatic diseases. Data for each subject who participated in the electrophoretic studies are being published in detail (6). The results obtained are in accord with those of other investigators and show that alterations of plasma protein *do* occur in uncomplicated and in complicated pregnancy. Gutman(7) has reviewed the difficulties of interpreting plasma protein levels in health and disease.

Materials and methods. Proteins were determined in the plasma of maternal venous blood obtained at or less than one hour preceding delivery and in the plasma of cord blood. Paired samples of maternal and cord blood were obtained from 11 women whose pregnancies were uncomplicated and from 21 women whose pregnancies were complicated by disease. Samples of venous blood were obtained by withdrawing 20 ml of blood from a vein in the antecubital fossa and discharging

TABLE I. Proteins in 11 Samples of Venous Blood at Delivery Following Uncomplicated Pregnancies, and in the Corresponding Cord Blood.

		Venous blood			
		g/100 g plasma protein		g/100 ml plasma	
		Mean	Range	Mean	Range
	Total protein			7.71	6.80–10.26
	Albumin	45	37 –49	3.47	2.51– 4.60
Globulins	alpha 1	6.3	5 – 7.3	.49	.40– .66
	" 2	12.6	9.4 –16	.97	.79– 1.32
	beta	18.3	16.2 –21.6	1.41	1.11– 1.93
	phi	9.5	6.2 –12.9	.73	.45– 1.07
	gamma	8.3	4.4 –12.3	.64	.32– .87
A/G ratio	plasma	.82	.59– .96		
	serum*	.99	.74– 1.10		
		Cord blood			
	Total protein			6.47	5.44–8.60
	Albumin	57.7	53.1 –64.1	3.73	2.95–4.57
Globulins	alpha 1	4.9	3.3 – 6.5	.32	.18– .55
	" 2	8.5	6.8 –10.2	.55	.38– .76
	beta	8.8	5.9 –10.6	.57	.37– .91
	phi	6.6	5.4 – 8.2	.43	.29– .65
	gamma	13.5	9.6 –17.8	.87	.60–1.16
A/G ratio	plasma	1.36	1.14– 1.79		
	serum*	1.61	1.35– 2.16		

* Calculated as [plasma protein minus fibrinogen].

the fluid into a 50-ml, glass-stoppered flask containing 3 ml of 2.5% sodium citrate in physiologic saline. At delivery the umbilical cord was cut between clamps within 3 minutes. The proximal clamp was released immediately to allow cord blood to flow freely (without stripping) into test tubes containing 2 ml of 2.5% sodium citrate in physiologic saline. All samples were prepared for analysis as described elsewhere(6). The electrophoretic patterns of the plasma proteins were obtained with the Longsworth(8,9) scanning mechanism and a Tiselius cell with a single, long center section. Most determinations were run for 3 hours at 1°C and a potential gradient of about 6 volts/cm. The buffer solution was 0.1 N with respect to sodium diethylbarbiturate and 0.02 N with respect to diethylbarbituric acid, pH 8.55, ionic strength 0.1. In earlier publications(1,2) results given were based on determinations of total protein by the gravimetric or micro-Kjeldahl methods and correction of plasma volumes for dilution and red cell shrinkage by the procedure employed by Longsworth *et al.*(10). Later, analyses of human blood in this laboratory under controlled conditions provided a factor with which to correct for the effect of the anticoagulant and in an effort to achieve greater consistency total protein values calculated from the total area of the electrophoretic pattern have been used(6).

Results. Mean and range values for total protein, albumin, 5 globulin fractions, and the albumin-globulin ratios in plasma of venous and cord blood from 11 women whose pregnancies were uncomplicated are given in Table I. The values for total plasma protein are appreciably higher for maternal than for cord blood. Per 100 g of plasma protein and per 100 ml of plasma, on the average greater amounts of albumin and gamma globulin were found in cord blood than in the corresponding venous blood, coincident with lesser amounts of alpha, beta, and phi (fibrinogen) globulins. The mean and range for the A/G ratio were much greater for cord than for maternal blood, portraying the higher level of albumin and lower level of total globulin in the cord blood. The patterns for cord blood corresponded more closely with those for healthy, nonpregnant women(6) than with those for maternal venous blood at delivery.

The average values (Table I) for the relative concentrations in venous blood at delivery and in cord blood, converted to "serum" values by subtracting the amounts of fibrinogen, compare favorably with results reported

TABLE II. Range Values of Protein Fractions in Venous and Cord Blood Following Uncomplicated and Complicated Pregnancies.

		Venous*		Cord			Venous*		Cord	
	Samples	Min	Max	Min	Max	Samples	Min	Max	Min	Max
		Plasma protein, g/100 ml plasma					Phi globulin, g/100 g plasma protein			
Normal	11	6.80	10.26	5.44	8.60	11	6.2	12.9	5.4	8.2
Mild pre-eclampsia	1†	7.34		5.27	5.66	2†	8.8	10.7	7	8.1
Severe ”	2	6.70	7.16	4.91	5.58	2	10.1	12.6	7.3	9.8
Essential hypertension	1	8.30		5.77		2	9	10.2	8.4	11.2
Renal disease	2	7.36	7.42	6	6.82	2	10.9	11.8	6.6	7
Diabetes	3†	6.24	6.40	4.78	5.84	3†	10.1	10.8	5.6	7.2
		Albumin, g/100 g serum protein‡					Alpha$_1$ globulin, g/100 g serum protein‡			
Normal	11	42.5	52.4	57.5	68.4	11	5.5	8.4	3.5	7
Mild pre-eclampsia	8	44	52.6	55.4	64.4	8	5.5	12.8	4	6.9
Severe ”	2	40.4	47.5	59.9	64	2	8.9	10.3	5.6	7.6
Essential hypertension	3	42.4	47.2	59	64.7	3	7.1	11.2	4.3	6.7
Renal disease	2	42.6	47.4	58.7	62.7	2	6.8	9.8	3.3	5
Diabetes	4†	47.2	53.1	61.2	63.6	4†	5.9	9.1	3.4	6.4
Rheumatoid arthritis	1	43.8		60.9		1	8.1		4.5	
		Alpha$_2$ globulin, g/100 g serum protein‡					Beta globulin, g/100 g serum protein‡			
Normal	11	10.4	17.5	7.2	10.9	11	18.2	23.7	6.3	11.5
Mild pre-eclampsia	8	14	29.7	7.7	13.1	8	9.3	21.3	5.9	13.7
Severe ”	2	14	18.5	8.4	12.3	2	19	20.1	11.2	13.3
Essential hypertension	3	11.8	18.4	8.1	10.6	3	16.3	25.3	8.1	10.9
Renal disease	2	15.7	17	9.7	12.1	2	20	22.7	7.1	9.4
Diabetes	4†	12.1	14.9	7.5	10.1	4†	17.4	21.9	7.8	12
Rheumatoid arthritis	1	13		8.3		1	18.7		8	
		Gamma globulin, g/100 g serum protein‡					Serum‡ A/G ratio			
Normal	11	4.7	14.1	10.2	18.9	11	.74	1.10	1.35	2.16
Mild pre-eclampsia	8	7.5	11.4	10.8	16.7	8	.78	1.11	1.24	1.81
Severe ”	2	6.1	15.2	6.9	10.8	2	.68	.90	1.49	1.78
Essential hypertension	3	8.4	13.4	12.4	14	3	.74	.89	1.44	1.83
Renal disease	2	8.8	9.2	15.5	16.5	2	.74	.90	1.42	1.68
Diabetes	4†	8.9	14	9.8	16.9	4†	.89	1.13	1.58	1.75
Rheumatoid arthritis	1	16.4		18.3		1	.78		1.56	

* Only venous blood samples for which a corresponding cord sample is available are included.
† One less sample for venous than for cord blood. Number given is for venous samples.
‡ Calculated as [plasma protein minus fibrinogen].

by Longsworth, Curtis, and Pembroke(10). The data of Longsworth *et al.* for alpha$_2$ globulin in venous blood were 19% lower and for beta globulin were 12% higher than values from the present study. Cord blood values for alpha$_1$ and alpha$_2$ globulins in this study were 12% lower than those of Longsworth *et al.* Other averages in the 2 studies agreed within 9%. The relative fibrinogen values given by Longsworth were 12% and 21% lower, respectively, for venous and cord blood than those given in Table I. The average total serum protein for maternal blood determined from the total pattern area by Longsworth *et al.* was 7.17 g per 100 ml, and in the present study was almost identical—6.98 g per 100 ml. The cord blood proteins also are very similar, 6.18 and 6.04 g per 100 ml. The A/G ratios approximate those reported by Longsworth *et al.*, and the relationship between the maternal and fetal ratios is similar. The ratios of gamma globulin to total globulin in the plasma of the two types of blood also were similarly related in the two studies. Although Lagercrantz(11) used a phosphate buffer at pH 7.7 and 0.15 ionic strength, the relative values for the proteins gave results similar to those reported by Longsworth *et al.* and in the present study.

Although the investigative procedure was designed to provide values for plasma, under some conditions imperfect mixing of the anticoagulant with the blood permitted clotting. Cord blood, especially, was subject to this hazard. Centrifugation at 12000 r.p.m. for 30 minutes removed the clot and cells, but only data for clot-free samples could be included in the ranges of values for plasma presented in Table I. Restriction of data for complicated pregnancy to clot-free plasma samples would have reduced even further the small numbers of samples in some groups. To avoid this, data are given in Table II on the basis of "serum" determined as total protein minus phi globulin. Minima and maxima are given for paired samples of maternal and cord blood from mothers with mild pre-eclampsia, severe pre-eclampsia, essential hypertension, renal disease, diabetes, and rheumatoid arthritis.

Venous and cord blood plasma from mothers whose pregnancies were uncomplicated (Table I) showed statistically significant differences in total protein, albumin, and the 5 globulin fractions, on the bases of protein distribution and of concentration per 100 ml of plasma. On the basis of plasma protein distribution there was a decided difference between the amounts of albumin in cord and venous blood, but on the basis of plasma concentration there was no marked difference between cord and venous blood. In complicated pregnancies (Table II) the ranges for total plasma protein both in venous and in cord blood were lower than those for the normal group: respectively, the maxima and minima were considerably lower. The maximum for cord blood total plasma protein in each group of complicated pregnancies also was lower than the minimum level for the corresponding average for venous blood.

For 11 samples of venous blood from mothers whose pregnancies were complicated, plasma fibrinogen values ranged from 8.8 to 12.6 g per 100 g of plasma protein, whereas in 12 samples of cord blood it ranged from 5.6 to 9.8 g and in the other sample—from a patient with essential hypertension—11.2 g per 100 g was found.

Ranges of values for the proteins of "serum" (plasma minus fibrinogen) from cord blood following complicated pregnancies were, in general, higher for albumin and gamma globulin and lower for alpha and beta globulins than the ranges found for venous blood. In neither venous nor cord blood were consistent differences discovered between data for complicated and uncomplicated pregnancy. In cord blood following both uncomplicated and complicated pregnancies the serum A/G ratios were almost double those of venous blood.

Discussion. Slemons(12) showed that nonprotein nitrogen concentrations of maternal and fetal blood were practically identical. The higher contents of amino acids and some crystalloids in fetal blood, according to Novak and Lustig(13), compensate for the greater amount of albumin in the maternal blood and keep the osmotic pressure about equal. Adaptation of the maternal and fetal osmotic pressures is considered essential(13) for the preservation of the vital nutritive chorionic epithelium of the fetal villi.

Recently, Moore, DuPan, and Buxton(14) determined electrophoretically the proteins in cord blood, in venous samples taken from the mothers soon after delivery, and in samples of blood taken throughout the gestation period and during the first year. They believe that the antibodies in newborn infant sera are a part of the gamma globulin but have little quantitative relation to it because they found gamma globulin in cord sera to be consistently higher than in maternal sera, whereas others had shown that antibody titers are never higher.

Longsworth, Curtis, and Pembroke summarized the results(15-17) of investigations of protein concentration per 100 ml of "serum or plasma" obtained by chemical methods and pointed out that "although the values obtained by different workers for a given type of material are not in quantitative agreement, the results of any one research are consistent in indicating certain systematic differences between bloods from the 3 sources." The electrophoretic results presented by Longsworth *et al.* supported the chemical results in the literature(15-17) and, in addition, revealed that although fetal serum is poorer in total globulin than is the maternal serum, it is relatively rich in gamma globulin, which is presumed to contain the major portion of antibodies.

Summary. 1. Total protein, albumin, and 5 globulin fractions were determined by electrophoresis in the plasma of samples of cord blood and of venous blood from 11 women whose pregnancies were uncomplicated. Similar samples were obtained for electrophoretic analysis from 21 women whose pregnancies were complicated by toxemia of pregnancy and other diseases. 2. For the samples representing uncomplicated pregnancy total protein values for venous blood were higher than for cord blood. Greater average amounts of albumin and gamma globulin were found in cord blood than in the corresponding venous blood, coincident with lesser amounts of alpha, beta, and phi globulins. 3. In complicated pregnancies the ranges for total plasma protein in venous and cord blood were lower than for the normal group. Ranges of values for serum protein (calculated as plasma minus fibrinogen) fractions in cord blood following complicated pregnancies were, in general, higher for albumin and gamma globulin and lower for alpha, beta, and phi globulins than the ranges for venous blood.

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Visualization of Arterio-Venous Shunts by Cinefluorography in the Lungs of Normal Dogs.* (19581)

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Catheterization of the pulmonary vessels in man and animals is currently a common procedure for experimental and diagnostic purposes. Frequently the catheters are advanced peripherally in branches of the pulmonary artery until they occlude the vessel in order to record "pulmonary capillary" pressure or to obtain arterialized blood samples by retrograde flow. In view of this technic and the many descriptions of arterio-venous shunts in

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the lungs of mammals it is of interest to report that following the occlusion or "jamming" of the pulmonary artery by a catheter, large A-V shunts can be visualized by cinefluorography. Since Prinzmetal *et al.*(1) have shown that glass spheres, many times the diameter of capillaries, will pass through the pulmonary vessels of the dog, cat, and rabbit, the presence of arterio-venous (A-V) shunts in mammalian lungs have received attention for their role in blood gas analyses(2), the passage of parasites through the lung(3) and the formation of arterio-venous aneurysms(4-7). Although A-V shunts were not observed in isolated human lungs by some investigators(8,9), they have more recently been demonstrated within the lobules and visceral pleura(10) by the use of the glass sphere technic of Prinzmetal *et al.*(1) followed by the injection of radiopaque media, vinyl acetate or liquid latex.

Method. Four dogs, with body weights of 40-55 lb, were anesthetized with Veterinary Nembutal (1 cc/5 lb). A Bard x-ray, whistle tip catheter, size No. 11, with openings at the tip and at 1 and 2 cm proximal to the tip, was passed into the jugular vein, right atrium and ventricle, and into the pulmonary artery. The progress and final position of the catheter were determined by fluoroscopic examination. During each cinefluorographic study, 20 cc of Thorotrast (24-26% ThO_2 by vol.) was injected in 2 to 3 seconds and 15 or 30 frames of 35 mm film per second were exposed for a period of 10 to 15 seconds.

Results. With the tip of the catheter in the pulmonary conus, the contrast medium passed into and through the pulmonary artery and veins, the left chambers of the heart and the aorta. But if the catheter was later advanced further (about 4 cm for dogs No. 1, 3, and 4) or retracted and then reinserted (No. 3) until "jammed" into the pulmonary artery of the left lower lobe, there was little filling of the branches of the pulmonary artery; most of the Thorotrast passed from one of the proximal openings in the catheter via an A-V shunt into the left chambers of the heart. In another instance (the second injection of dog No. 4) when an attempt was made to withdraw the catheter, arterial spasm held its distal end firmly but after several forceful trials, the catheter was dislodged and withdrawn until its tip was in the pulmonary conus. The injected Thorotrast in this case refluxly filled both major branches of the pulmonary artery and its smaller branches. Although the shunt could not be visualized with certainty, the left atrium contained some radiopaque medium before the remainder had traversed the pulmonary veins, indicating that the shunt must have remained open even though the catheter was removed from the pulmonary artery.

In one dog (No. 2) no evidence of A-V shunts was noted. The Thorotrast coursed normally through the pulmonary vessels of this animal both when the catheter tip was 4.5 cm out from the pulmonary conus and one-half hour later, when it was advanced about 2.2 cm still further.

Additional confirmation of the A-V shunt was obtained in dog No. 1 when one catheter was inserted into the pulmonary conus and another passed retrogradely through the femoral artery into the aorta, until the tip of the latter was just above the semilunar valves (dog No. 1). While this dog was breathing 30% O_2, several hundred glass spheres, 200 $\pm$ 25 μ in diameter, in saline were injected via the catheter into the pulmonary artery. Three seconds after introducing the spheres, 10 cc of arterial blood was withdrawn as rapidly as possible from the aortic catheter and one glass sphere was recovered. Repeating this procedure, but with the animal breathing 10% O_2, 2 spheres were recovered. This dog was killed and the heart and lungs removed as a unit. The pulmonary arteries were injected with red, and the veins, with blue vinyl acetate, respectively. The casts of the pulmonary vessels were obtained according to the technic of Tobin and Zariquiey(10) and revealed the presence of an A-V shunt in the inferior lobe of the left lung.

Discussion. In a routine study of the anatomy of pulmonary vessels of dogs by cinefluorography it was discovered that A-V shunts in the lung could be readily visualized when the catheter was advanced until it "jammed" in the pulmonary artery. In 12 dogs with the catheter tip in the right atrium, ventricle, pulmonary conus or the proximal part of the pulmonary artery, no evidence of A-V shunts was ever seen with the injection of Thorotrast. However, with deliberate "jamming" of the catheter into a branch of the pulmonary artery, A-V shunts were seen in 3 of 4 dogs. It is suggested that the irritation produced by the catheter may cause the pulmonary vessels to constrict around the catheter as well as peripherally and thus force the injection material through A-V shunts. Another explanation is that this irritation may cause the A-V shunts to open without need of greater filling pressure.

Summary. A shunt between the pulmonary artery and vein was demonstrated in 3 of 4 dogs by injecting Thorotrast into a catheter inserted tightly into the pulmonary artery and following the circulation by cinefluorography. In one of these dogs the passage of glass spheres 200 ± 25 μ in diameter, through a cannula in the pulmonary artery, to and through a cannula in the aorta, together with the location of the shunt from plastic casts of the pulmonary vessels, help to confirm the presence of the shunt.

The authors are greatly indebted to Dr. J. S. Watson and Mr. S. A. Weinberg of the Department of Radiology, for their aid in the cinefluorography. This technic has been previously described(11).

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Absorption Rates of Colloid and Milky Suspensions of Halogenated Fats for Hepatosplenography in the Rat. (19582)

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Success with intravenous injections of emulsified iodized oils for hepatosplenography has not been achieved, partially because of the pyrogenic effect of the fat and toxicity of the halogens(1). This report concerns attempts to lessen these toxic effects by the use of injection methods other than by the intravenous route.

Experimental. Milky and colloid suspensions of the following halogenated fats were used in these experiments: 40% iodine in poppy seed oil (Lipiodol LaFay), 27% iodine in peanut oil (Iodochloral Searle), bromiodized linseed oil, iodized linseed oil, brominized linseed oil, bromiodized lecithin and hexabromostearic acid. Owing to the fact that the reaction between the halogen and the nonsaponifiable portion of fat seems to produce toxic substances, recrystallized hexabromostearic acid was tried. This was prepared by dissolving 2 g (M.P. 179-180°C) in 10 ml of boiling dioxane which was then injected as a capillary stream into 15 ml boiling distilled water rotated in a micro Waring blendor. The resulting sol was bluish by reflected and reddish by transmitted light. After stabilizing with 1% polyoxyethylene sorbitan-fatty esters (Tween 80), it was dialyzed free of dioxane. In other experiments, Lipiodol as well as bromiodized and brominized oils were emulsified with 4 volumes 1% Tween 80 in a 2-stage recirculating homogenizer at a pressure of 3000 lbs/sq in. These emulsions contained droplets of 1 to 3 μ and probably invisible particles of colloid size. In some experiments, we used colloid sols of Lipiodol showing the Tyndall phenomenon, obtained by using larger amounts and higher concentrations of Tween 80.

Most of the emulsions used in these experiments were injected either into the subcutaneous, muscular, perivenous lymphatic spaces, or stomach. In some experiments, a combination of these injections with the intravenous route was tried. For the perivenous lymphatic injections, the loose areolar tissues around such large veins as the jugular or femoral veins were selected. Absorption from these spaces was watched in preliminary experiments by using emulsions of corn oil stained with Sudan Black B. By sacrificing animals at various periods, these emulsions could be traced into the regional lymph nodes and then into the general circulation at 4 hours to 5 days after injection. A few experiments were done after intrapleural and intraperitoneal injections. An ampule of hyaluronidase 150 TR units (Widase) was mixed with each 10 ml of emulsion to speed absorption of all the subcutaneous and intramuscular injections. In the emulsions given by stomach tube, 10 mg procaine were added to open the pylorus. Rats weighing 200 g were used in all experiments.

Results. Successful hepatosplenography was not obtained with a single dose of the tested emulsions given subcutaneously, intramuscularly or by stomach tube alone. A slight accentuation of the liver and spleen shadows was obtained if the emulsions were given repeatedly by stomach tube. The absorption of halogenated sols from the pleural and peritoneal cavities was too slow to be of any value(1). In Table I are recorded the results obtained with the intralymphatic and intragastric injections alone and combined with intravenous injection.

TABLE I. Hepatosplenography with Various Iodized, Bromiodized and Brominized 20% Emulsions in Tween 80 after Intralymphatic and/or Intragastric Injections or Combined with Intravenous Injections (in ml Doses).

No. of rats	Intra-lymphatic	Intra-gastric	Intra-venous	Hepatosplen-ography
5	12	—		—
3*	—	40		+
5	3	5		—
50			3	++
5			1.5	—
5		5	1.5	+
5	3	5	1.5	++
3†		8	3	++

Accentuation of liver or spleen x-ray shadow: — none; + slight; ++ moderate.

* 10 ml doses at 9 a.m. and 3 p.m. for 2 days.

† Intravenous dose 5 days before intragastric dose.

Successful hepatosplenography with any emulsion was never obtained with less than 3 ml. With this dose, the hepatic and splenic shadows were well intensified within 1 to 2 hours after intravenous injection. These shadows faded within 5 to 7 days; but if then another injection were given either by the gastric or intralymphatic routes, the hepatic and splenic shadows again were potentiated, in spite of the inadequacy of the intragastric or intralymphatic dose given alone.

With combinations of different routes of injections, it was found that good hepatosplenography was obtained 5 hours after intralymphatic and 24 to 30 hours after intragastric if an intravenous injection were given one hour before x-ray studies. Dibromindigo appears in the urine after intragastric injection of hexabromostearic acid, suggesting that the halogen is partially split off in the alimentary tract.

Discussion. The rapid subcutaneous absorption of fat emulsions observed by Shafiroff (2) seems not to apply to halogenated fats for purposes of splenography. Nor were we able to obtain the results of Olsson and Ekman(3) with single intragastric injections. The combination of routes with the intravenous injection, in spite of good results with smaller doses, does not seem practicable for use in the human. No studies of the storage capacity of our emulsions were done, but the darkening of halogenated fats in light is apparently hastened by the non-saponifiable portion and the emulsifier.

Boiling emulsions for sterilization was never done without an increase in size or aggregation of droplets; but boiling for sterilization was found to be not necessary. Tests with broth and blood agar plate cultures for bacteria and Littman plate cultures for molds were found negative. Apparently, the halogen acts as a sterilizing agent.

Summary. 1. A colloid sol of hexabromostearic acid was used successfully for hepatosplenography in the rat. This sol could be given intravenously or by repeated intragastric injections. Dibromindigo appeared in the urine after intragastric injection. 2. Half the minimum intravenous dose of several halogenated fat emulsions for hepatosplenography was effective when reinforced with injections by the intralymphatic and intragastric routes, but the total dose was greater than the minimum intravenous dose alone. 3. The emulsions were broken by boiling but this method of sterilization was not necessary since it was found that the halogens acted as sterilizing agents.

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Combination of Hydrochloric Acid and Sodium Hydroxide with Human Aortic Albuminoid.* (19583)

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Recent studies have revealed that the albuminoid (collagen and elastin) fraction of the human aorta undergoes significant quantitative aging and pathological changes. Though the percentage of elastin in this albuminoid fraction increases significantly only in markedly arteriosclerotic aortae(1), notable increases in the calcium content of aortic elastin have been demonstrated with progressive age(2). Likewise, important quantitative aging changes in the amino acid composition of elastin have been shown(3). It seemed of value, therefore, to investigate the acidic and basic properties of the human aortic albuminoid as part of an overall attempt to more fully evaluate the underlying pathological changes in this tissue.

Materials and methods. The albuminoids were prepared by previously described methods(4) with the exception that sodium chloride and dibasic sodium phosphate soakings were substituted for the calcium hydroxide treatments. The refrigerated tissues, subsequent to acetone and alcohol extractions, were soaked in 3 changes of 10% sodium chloride for a minimum of 24 hours during each extraction period. They were then treated with M/15 dibasic sodium phosphate for 2 soaking periods of at least 24 hours in the refrigerator. After five 24-hour washings in distilled water, the samples were dehydrated with several changes of absolute alcohol, dried in an oven at approximately 70°C, and desiccated for at least 24 hours. The 29 albuminoid specimens were each subdivided into 2 approximately equal samples ranging from 0.4 g to 2.6 g in weight. One aliquot of each tissue was treated with 30 ml of .10 N hydrochloric acid in saturated sodium chloride (25°C) per g of albuminoid, while the other fraction was soaked in 30 ml per g of tissue of .11 N sodium hydroxide in saturated sodium chloride (25°C) according to the method of Beek(5). After standing for 24 hours with occasional shaking,

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TABLE I. Combination of Hydrochloric Acid and Sodium Hydroxide with the Human Aortic Albuminoid.

Age group, yr	No. of samples	Mean mEq HCl combined/g albuminoid	Stand. dev.	t	P	Mean mEq NaOH combined/g albuminoid	Stand. dev.	t	P
0-39	7	.77	.10			.76	.05		
40-59	5	.95	.31	1.4	.18	.81	.02	1.8	.09
60-74	8	1.3	.48	3	.01	.79	.03	1.2	.24
75-90	9	1.7	.40	6.1	<.01	.79	.02	1.7	.11
Condition*									
+	4	.77				.78			
2+	4	.77				.75			
3+	5	1.1				.81			
4+	9	1.3				.79			
5+	7	1.8				.79			

* The aortae were divided on the basis of gross observations into five groups: Normal (+), few atheromatous plaques (2+), moderate atheromatous plaques (3+), atheromatous plaques and few calcified plaques (4+), and many plaques, atheromatous and calcified (5+).

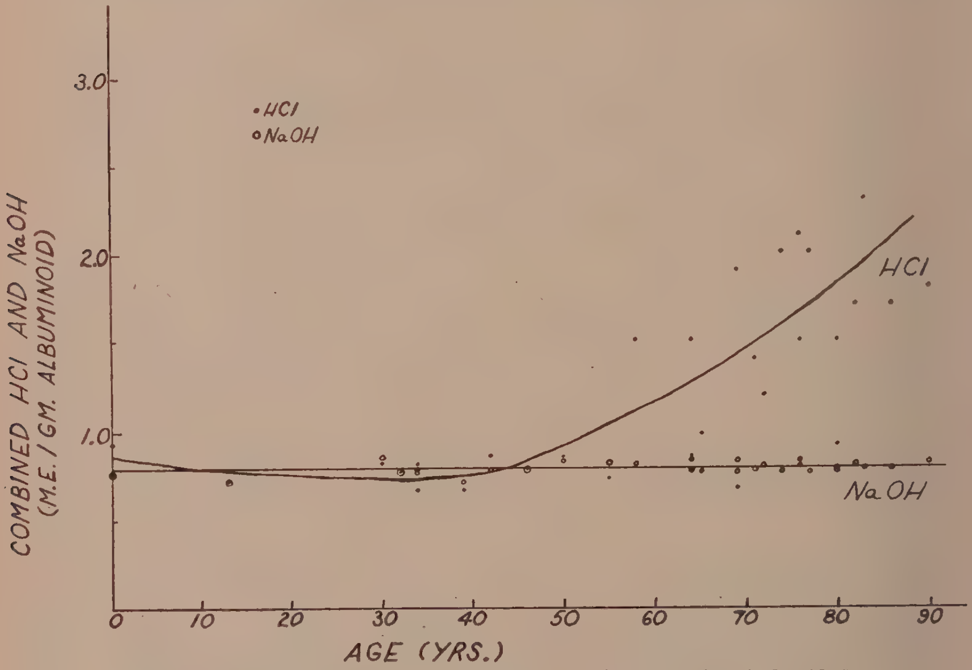

FIG. 1. Aging changes in combined hydrochloric acid and sodium hydroxide.*

a 5 ml aliquot of each supernatant solution was titrated against .10 N hydrochloric acid or .11 N sodium hydroxide, using methyl red as the indicator. All samples were treated simultaneously at a temperature of 26 ± 2°C. The results were expressed in terms of the number of milliequivalents of acid or base combined by one g of albuminoid.

Results. Aging changes. All samples combined with both sodium hydroxide and hydrochloric acid. The quantity of combined sodium hydroxide per unit weight of tissue remained constant in all age groups (Table I, Fig. 1). However, an approximately 2-fold increase in the quantity of combined hydro-

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* Plotted by visual approximation.

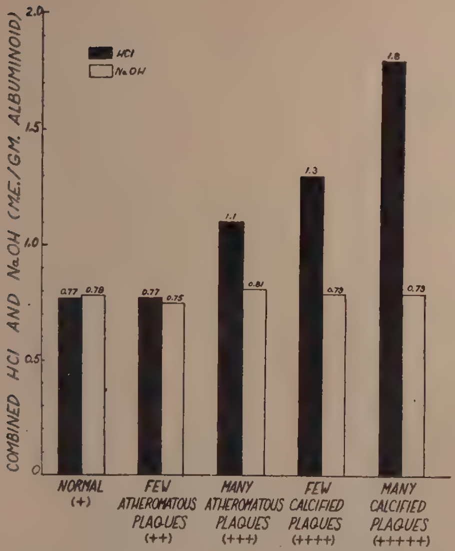

FIG. 2. Pathological changes and combined hydrochloric acid and sodium hydroxide.

chloric acid was observed to take place progressively between the ages of 40 and 90 years (Table I, Fig. 1). The statistical analyses (Table I) indicate that the increase in combined hydrochloric acid in the 60- to 90-year age groups is statistically significant, when compared with normal values. There are, however, no statistically significant age changes in the quantity of combined sodium hydroxide.

Pathological changes. The samples have been arbitrarily classified into 5 groups on the basis of gross pathological findings (Table I, Fig. 2). The amount of combined sodium hydroxide remained relatively constant in all groups. Markedly sclerotic aortae combined with larger amounts of acid than did normal or mildly atheromatous samples, while moderately sclerotic tissues yielded intermediate values. The chi square test showed that the differences in the number of milliequivalents of combined hydrochloric acid per gram of tissue for the various pathological groups cannot be accounted for by chance alone ($P = < .01$).

Discussion. If it is assumed that the calcium present in these tissues is in the form of tricalcium phosphate(3), this salt would certainly be an important factor in determining the above results. In addition, the presence of other minerals as well as the acid and base combining power of the protein itself must be taken into consideration. It is also difficult to determine whether the increased mineral deposition in the aorta with aging and pathological changes is due to changes in the albuminoid proteins themselves, or whether the protein changes are secondary to the increased mineral deposition.

Summary. 1. Human aortic albuminoid fractions combine with equal amounts of sodium hydroxide despite differences in age or pathological condition. 2. Arteriosclerotic and aged aortic albuminoids combine with larger quantities of hydrochloric acid than do relatively normal or younger samples.

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Direct Isolation of *Histoplasma capsulatum* from Soil: Probable Etiological Relationship to Camp Gruber Pneumonitis. (19584)

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From the Communicable Disease Center, Public Health Service, Federal Security Agency.

No reports of the direct isolation of *Histoplasma capsulatum* from the soil have appeared in the literature. Emmons(1), and Ajello and Zeidberg(2), and the authors(3) have been successful in isolating it indirectly from the soil by modification of the flotation method of Stewart and Meyer(4) combined with animal inoculation. Grayston, Loosli, and Alexander(5) similarly isolated it from mixed sawdust and soil from an unused silo. Attempts by these authors to isolate the organism directly by culture of these materials were unsuccessful. Streaking the dry material or using a portion of the sample prepared for animal inoculation on culture plates always resulted in the plates being overgrown with saprophytic fungi and bacteria. The present paper reports the first direct isolation from the soil.

Both the direct and indirect isolations of *H. capsulatum* reported in this paper were made from soil collected from an abandoned storm cellar at Camp Gruber, Okla. This storm cellar was entered on Mar. 17, 1944 by some 26 soldiers, all of whom subsequently developed a severe pneumonitis. The characteristics of this epidemic have been reported by Cain, Devins, and Downing(6), and Mickle (7). Epidemiological and case history studies revealed that the time of exposure of these men ranged from 3 minutes to 6 hours. Due to the cold weather prevailing at the time a fire had been built in the cellar by the men who chipped bark and wood chips from the elm tree posts supporting the roof and prying loose rotten boards from the ceiling. The widespread lung lesions which developed in the men exposed suggest that the infection was inhaled in the storm cellar.

Soil samples were collected on 4 different occasions from various locations in the interior of the storm cellar. These collections were made in July 1950, and in February, May and June 1951. The soil was placed in sterile quart Mason jars and transported to the laboratory for study. The lids were screwed lightly to permit at least partial aeration. The samples were stored in a room in which no cultures of *H. capsulatum* were maintained.

The direct isolation was made from soil collected in Feb. 1951. A modification of the Emmons' flotation method(1) was used. Twenty cc of soil were added to a sterile 100 ml glass-stoppered graduated cylinder. Thirty cc of sterile saline were added to the soil and the mixture was shaken vigorously for 2 or 3 minutes and then allowed to stand undisturbed. Samples were removed from these graduated cylinders from different depths of the supernate after 30 minutes, 1 hour, and 1½ hours. Varying amounts of penicillin, streptomycin and actidione were added to the supernate before plating on various media. Combinations of 2000, 4000 and 8000 units of penicillin and streptomycin and 0.1 mg to 1.6 mg of actidione per ml of the supernate were used. These antibiotics were also incorporated into the numerous selective media which were utilized. These media and antibiotics were tested previously to determine that they had no inhibitory or retarding action on 21 isolates of *H. capsulatum*.

A medium developed in our laboratory was the substratum on which the primary isolation of the fungus was made. This medium was made from an extract of fresh elm bark (*Ulmus americana*) obtained from recently felled American elms. The bark was cut into strips and separated into inner and outer portions. The inner portion, which consisted of both functional and nonfunctional phloem was the only material used in the preparation. One thousand g of the stripped and shredded bark were immersed in distilled water and brought to a boil for a period of 3 hours. The elm bark extract was then made up to a volume of 5 liters by the addition of distilled water and filtered through coarse filter paper (E & D 615). Since the pH of the water extract was usually near 3.5, it was adjusted to 6.8 with a buffered solution (phosphate buffer) before adding agar. Twenty-five cc portions of the medium were dispensed into 160 ml medicine bottles and autoclaved at 121°C and 15 lb pressure for 15 minutes. It was then slanted in the bottles and cooled.

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Nearly 400 attempts were made before the fungus was finally isolated from a single culture on the elm bark medium. This positive culture was obtained from the interfacial area between the supernate and the sediment of the soil sample. In nearly every case when preparations of the supernate from this region were examined microscopically, spores characteristic of *H. capsulatum* could be observed. The isolate was inoculated into mice and into 2 white rats. When the organs of these animals were aseptically macerated and streaked on various media the fungus was recovered. The blood sera of both rats showed a positive complement fixation test for histoplasmosis 99 days after infection. The isolate was also converted to the yeast phase on brain heart infusion blood agar at 37°C.

H. capsulatum was isolated following mouse inoculation from the samples collected in June 1950, and in Feb. and June 1951. The following method was employed: 10 cc soil were placed in sterile 100 cc graduate and saline added to 100 cc. After 5 minutes of shaking, the sample was allowed to stand 30 minutes. All the clearest supernatant fluid (usually about 75 to 80 cc) was drawn off with a sterile pipette and put in sterile tubes. These tubes were centrifuged at 2500 r.p.m. for 30 minutes. The supernate was discarded and the lower 2 or 3 cc in each tube were combined to make a volume of 15 cc. Eight thousand units of streptomycin and penicillin were added to each cc. One cc was inoculated intraperitoneally into white mice.

The organism was isolated from one of 9 mice inoculated with the June 1950 soil, 2 of 14 mice inoculated with the February 1951 soil, and one of 2 mice inoculated with the June 1951 soil.

In addition to the one direct isolation of *H. capsulatum* and 3 isolations following animal inoculation with the soil of the cellar in which the pneumonitis was contracted, other evidence that the organism is etiologically related to the pneumonitis is available from follow-up of the soldiers involved in the epidemic. While these results will be reported in full later, it appears worth while to summarize them briefly here. Follow-up studies were obtained in 1951 of 23 of the 26 men involved in the 1944 epidemic at Camp Gruber, and of all of 8 men who were in the same organization as the men involved in the epidemic, but who remained well. These men served as controls. The studies were undertaken with the cooperation of the Commission on Acute Respiratory Diseases of the Armed Forces Epidemiological Board.

The results of the follow-up studies show that all of the 22 men tested who were involved in the 1944 pneumonitis epidemic reacted to histoplasmin, while only 6 reacted to tuberculin, 3 to blastomycin, and one to coccidioidin. Among 8 control persons of the same organization who did not become ill, 4 of 8 reacted to histoplasmin. Three of these gave a history of brief exposure in the suspected storm cellar. Four also reacted to tuberculin, one to coccidioidin, and none to blastomycin. Since all of the men involved had spent all of their non-Army residence in areas where the skin test sensitivity to histoplasmin is low, it appears that their skin reaction to this antigen is probably connected with their Army service.

In addition to skin tests, follow-up blood complement fixation tests for histoplasmosis were performed. Four of the 20 pneumonitis cases showed a positive test, whereas none of the 8 controls showed such a result.

The most dramatic evidence is shown in the x-ray picture. Disseminated calcifications were seen in the chest x-rays of 16 of the 22 men who had pneumonitis at Camp Gruber while such calcifications were found in none of the 8 controls. Since White and Hill(7), and Furcolow(8) have reported that over 95% of 298 cases of disseminated calcification showed a positive histoplasmin skin test, and since Furcolow(9,10) has followed 2 cases of disseminated lung lesions due to proved histoplasmosis from the acute lesions to final disseminated calcification, this furnishes strong supporting evidence of the etiological relationship.

Summary. We report the first direct isolation of *H. capsulatum* from natural sources; *i.e.*, soil from a storm cellar in which severe pneumonitis was contracted by soldiers in 1944. This organism was also isolated from soil from the storm cellar following animal inoculation on three occasions. This evidence and that obtained by studies of the men involved in the pneumonitis epidemic indicates that the epidemic was caused by H. *capsulatum.*

The authors wish to thank Dr. Charles E. Smith, University of California School of Public Health, Berkeley, who was the first to suggest histoplasmosis as the probable etiological agent in this epidemic due to the very suggestive serological results obtained by him with sera from the patients in 1944.

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Effect of Deficiencies of Sodium and Potassium on Motility of the Intestine of the Rat.* (19585)

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Workers in this laboratory frequently have observed that animals receiving potassium deficient diets had distention of the stomach and of the small intestine. Also, many rats which were fed a potassium deficient diet containing 19% cellulose died of intestinal obstruction. These are evidences of decreased intestinal tone and motility.

Gazes *et al.*(1) reported that isotonic potassium chloride administered intravenously stimulated intestinal motility in human beings and dogs with adynamic ileus. Webster *et al.* (2) observed that rats fed a diet containing 0.01% potassium exhibited decreased intestinal tone and finally died of a condition resembling paralytic ileus. Since the observations of these workers were essentially qualitative, quantitative observations of the effect of dietary potassium deficiency on intestinal motility are of interest. The possible interrelationship of sodium with potassium in this regard also was investigated since increased levels of sodium have been shown to increase the mortality and the severity of the heart lesions of potassium deficient rats(3).

Experimental procedure. Groups of 5 adult female albino rats weighing from 180 to 220 g each were placed on each of the 4 experimental diets employed. The diets were essentially the same as the low sodium and potassium diets of Grunert and Phillips(4). The deficient diets contained 0.005% sodium when sodium was deficient and 0.005% potassium when potassium was deficient. The control diet contained 0.25% sodium and 0.5% potassium. After 6 weeks on the experimental

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TABLE I. Summary of Results.

Na and K content of diet		Distance bolus was propelled in 10 min		% of length of small intestine bolus propelled in 10 min	
% Na	% K	Avg, cm	Range	Avg	Range
.005	.005	49	45.7– 50.8	45.2	41.8– 48.7
.25	.005	49	43.3– 53.3	50.9	46 – 58.8
.005	.5	101	97.7–104	97	95 – 98
.25	.5	104	93 –105	98	97.5–100

diets the propulsive motility of the intestine of each of the rats was determined by means of a modification of the technic of Macht(5). The procedure used was as follows: The rats were fasted for 24 hours and then each rat was given 2 ml of a 10% suspension of charcoal in a 10% aqueous solution of gum acacia by stomach tube. No anesthesia was used at any point in the experiment. Exactly 10 minutes after the administration of the charcoal and gum acacia each of the rats was killed by a blow on the head followed by exsanguination. The small intestine was then dissected out from the pyloric sphincter to the ileo-cecal junction, laid out in a straight line, and its length and the maximum progress of the charcoal from the pyloric sphincter measured. The exsanguination and dissection took about 30 seconds.

Results. Potassium deficiency or a combined deficiency of sodium and potassium lowered the propulsive motility of the small intestine of the rats by about 50% under the conditions of this experiment (Table I). Sodium deficiency alone was without effect on intestinal motility as determined by this technic.

Discussion. Fenn(6) states that although potassium is not regarded as a humoral agent for neuromuscular transmission, it plays an important role at the neuromuscular junction. Since the peristaltic contractions of the small intestine are dependent upon the intrinsic nerve plexuses of Auerbach and Meissner(7), a decrease in the potassium content of the serum or of the muscle cells may cause a decrease in peristaltic activity by affecting the normal function of the neuromuscular junction. Meyer *et al.*(3) have reported a decrease in serum potassium in rats which received a diet containing 0.005% potassium and 0.25% sodium, but rats receiving a diet containing 0.005% potassium and 0.005% sodium did not exhibit hypopotassemia. In view of the decrease in propulsive motility of the intestine of the rats which received the diet containing 0.005% potassium and 0.005% sodium, serum potassium concentration does not appear to play a direct role in this phenomenon. The potassium content of visceral muscle may affect the neuromuscular junction or it may affect the ability of the muscle cells to respond to stimulation by affecting the enzyme systems involved in muscle contraction. Thus, potassium is known to be necessary for the phosphorylation of creatine in connection with pyruvate oxidation *in vitro*(8) and potassium deficiency may affect the functioning of this enzyme system *in vivo*.

Summary. Maintenance of adult female albino rats on a diet containing 0.005% potassium for 6 weeks resulted in a 50% decrease in intestinal propulsive motility. Reduction of the sodium content of this diet from 0.25% to 0.005% did not result in an increase in intestinal motility. Feeding a diet containing 0.005% sodium and 0.5% potassium did not alter the intestinal propulsive motility of the albino rats when compared with observations of control animals on a diet containing 0.5% potassium and 0.25% sodium.

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Vitamin B_{12} Biosynthesis after Oral and Intravenous Administration of Inorganic Co^{60} to Sheep.* (19586)

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The literature through 1950 concerning the chemistry, physiological role, and clinical use of vit. B_{12} has been reviewed comprehensively by Smith(1), Ungley(2), and Marston(3). The essentiality of cobalt in ruminant nutrition has been recognized for many years(4,5) and a number of distribution studies using inorganic radioactive cobalt have been reported(6-12). Also, some information is available regarding the distribution of vit. B_{12} labeled with radioactive cobalt(12-14). Nevertheless, the relationship between the dietary need of inorganic cobalt and the physiological role of vit. B_{12}, especially concerning the factors involved in B_{12} biosynthesis, has remained little understood.

The present work extends the information obtained in earlier radiocobalt studies by partition of the labeled cobalt into 3 distinct fractions of the excretions and tissues—namely, vit. B_{12}-like, inorganic, and bound. These data have yielded evidence concerning the degree and possible sites of vit. B_{12} biosynthesis in sheep.

Methods. Treatment of animals. Four normal wether lambs weighing about 80 lb each were placed in metabolism stalls. Each of the animals received 2 mc of Co^{60} chloride solution containing about 1.6 mg of cobalt. Two were dosed orally by gelatin capsule and 2 received an intravenous (jugular) injection. The metabolism stalls and the technics for handling the animals, administering the activity, and collecting samples have been described by Hansard, *et al.*(15). *Collection of samples.* Feces and urine were collected continuously and samples were taken daily over a period of a week. The urine was collected under toluene and both feces and urine samples were refrigerated with toluene added. At the end of 7 days the animals were sacrificed and various tissues were sampled for analysis. Aliquots of each sample were counted for total radioactivity. In addition, butanol-soluble and water-soluble phases of similar aliquots were separated and counted as described in detail below. In all samples except urine some residual activity was also obtained in the insoluble fraction.

Counting technics. Several counting technics were employed in the work, depending on the sample size and the amount of radioactivity present in the sample. With large amounts of activity, accurate counts were obtained by measuring a few grams of fresh sample using an unshielded Geiger-Müller tube with an 89.5 mg/cm^2 aluminum absorber, thus recording only gamma activity. With somewhat less activity, it was necessary to count without an absorber. In this case, a wet ash digest was prepared, to assure reproducible geometry, and measured with the unshielded thin-window tube. Still smaller amounts of activity were counted by using dry ashed samples and a shielded thin-window G-M tube.

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† Oak Ridge Institute of Nuclear Studies Fellow on leave from Alabama Polytechnic Institute.

When only minute amounts of activity were present, dry ashed samples were counted in a windowless proportional counter. Data from all methods were reduced to a comparable basis by counting standards from the original dosing solution under each set of conditions and calculating all values in terms of percent of the dose.

Preparation of samples for total radioactivity determinations. The total Co^{60} present in feces was usually determined by counting gamma radiation in 5 to 10 g of the fresh material. In a few samples, however, the count was low enough that solution counting was necessary. For this purpose about 5 g of feces were digested with concentrated nitric acid, the resulting solution made up to 10 ml and transferred to a 50 mm Petri dish for counting. In all urine samples, total radioactivity determinations were made by direct solution counting. For the determination of total activity in tissues, 1-3 g samples were placed in flat, 40 mm, capsule type porcelain crucibles, dried in an oven at 110°C and then ashed at 550°C in a muffle furnace for several hours. The ash was dissolved in a few drops of concentrated HNO_3 and enough water was added to form a thin layer over the bottom of the crucible. The samples were then evaporated to dryness under infra-red heat lamps and counted with a shielded thin-window G-M tube. Self-absorption corrections were made when the mass of the sample to be measured was large enough to produce more than a 5% loss of counts.

Chemical fractionation. The methods used in obtaining Co^{60} fractions from samples were developed from those discussed by Woodruff and Foster(16). For fractionating feces, 5 g samples were homogenized in a Waring blendor with 40 ml of 0.5% sodium phosphate buffer (pH 8.0), 1 ml of toluene, and 20 mg of crystalline trypsin. For tissues, 1 g samples were homogenized in a Potter-Elvehjem homogenizer with 10 mg of crystalline trypsin, a few drops of toluene, and phosphate buffer added to total about 8 ml. After incubation at 37°C for 45 to 120 hours, the homogenates were filtered or centrifuged. The residues were washed thoroughly and the washings added to the supernatant solution. The supernatant was saturated with ammonium sulfate and then extracted four times with equal volumes of *n*-butanol. It was found that under these conditions at least 95% of the butanol soluble Co^{60} was extracted into the butanol phase, while all the water soluble Co^{60} remained in the aqueous phase. The two phases were separated by centrifugation and subsequent removal of the butanol layer with a vacuum siphon. The Co^{60} in the butanol phase will be referred to throughout this paper as "vitamin B_{12}-like." When vit. B_{12} is mentioned without modification, it should be remembered that this is the fraction meant. Actually, paper chromatography of B_{12}-like extracts from several representative samples showed a behavior similar to a solution of pure B_{12} labeled with Co^{60}[‡] as contrasted with a solution of inorganic Co^{60}. The Co^{60} in the aqueous phase will be called "inorganic;" that remaining in the residues, "bound." Due to difficulties in manipulation the "bound" values were determined routinely by difference. However, some residues were counted directly and the values obtained agreed closely with those obtained by difference. No residue remained in the urine samples after extraction, which was carried out without preliminary trypsin digestion. Aliquots of all the above butanol and aqueous extracts were dried in 1 inch stainless steel cups, ashed at 450-550°C for an hour or two to reduce the mass, and counted in a windowless proportional counter.

Results and conclusions. In order to avoid confusion, it should be made clear at the outset that the data may be expressed by two distinct quantities: a) a percentage of the dose (*e.g.*, 84.4% of the ingested Co^{60} was excreted in the feces), and b) the percentage of Co^{60} in any given fraction as a function of the total Co^{60} present (*e.g.*, 12% of the Co^{60} found in the feces was vitamin B_{12}-like). The former quantity is used primarily in discussing total amounts of Co^{60} excreted or the concentration in tissues when expressed as percentage of dose per gram of fresh sample; the latter is used in expressing the relationships

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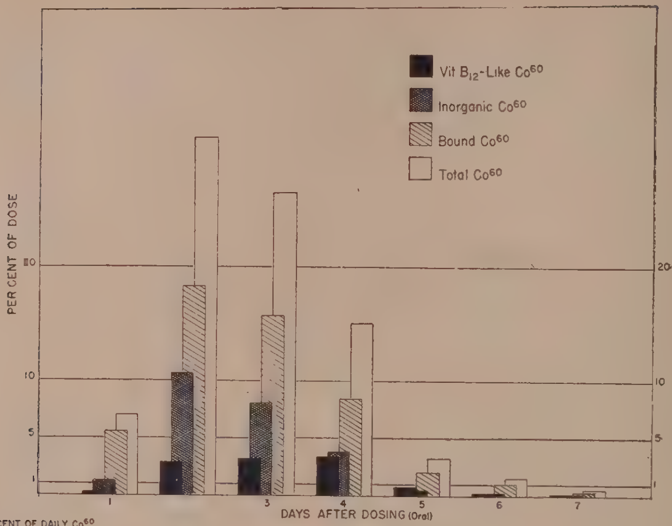
[‡] The Co^{60}-labeled vit. B_{12} was supplied by Merck & Co., Rahway, N. J.



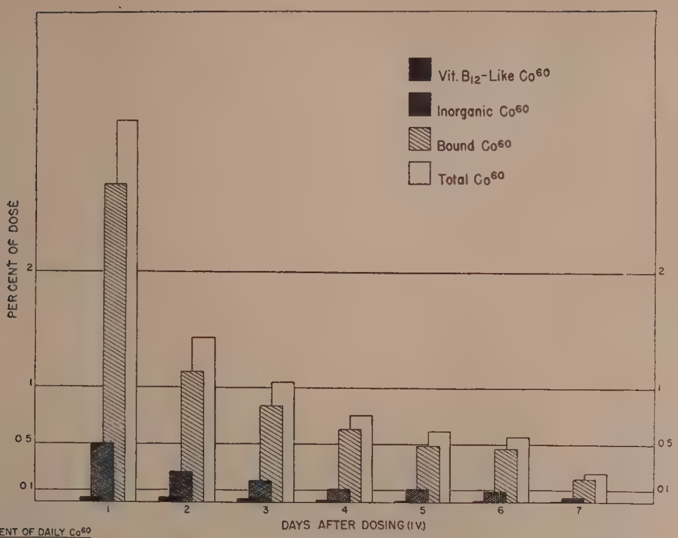
PER CENT OF DAILY Co^{60}	1	2	3	4	5	6	7
Total Co^{60}	100	100	100	100	100	100	100
Bound Co^{60}	79.8	57.1	59.0	55.6	64.0	70.3	57.2
Inorganic Co^{60}	17.4	34.0	30.0	24.2	12.9	13.3	20.0
Vit B_{12}-Like Co^{60}	3.8	8.9	11.0	20.2	23.1	16.4	22.8

FIG. 1. Average excretion of Co^{60} in the feces of two sheep after oral administration plotted as % of dose/day; the tabulated values indicate the % of total Co^{60} represented by each fraction. The total fecal excretion for the week amounted to 84.4% of the dose.

of the various fractions to one another and to the total amount of Co^{60} present. Average values for the 2 animals are presented in each case since they were in general agreement.

Total excretions. From Fig. 1 and 2 (feces data) and Table I (urine data) it may be noted that 84.4% of the oral cobalt dose was excreted in the feces and 10.8% in the urine. After intravenous injection 8.0% of the dose appeared in the feces and 77.7% in the urine. This small retention and dependence of excretory route upon the mode of administration is in general agreement with values reported for other species(6-11).

Fecal excretion. The details of Co^{60} excretion in the feces after oral administration are presented in Fig. 1 and after intravenous injection in Fig. 2. The graphs denote the percentage of administered Co^{60} excreted daily in each of the chemically characterized fecal fractions. For convenience, values for Co^{60} in each fecal fraction as a percentage of the daily total of Co^{60} in the feces are tabulated directly below the bars to which they correspond. The scale for Fig. 2 is one-tenth that for Fig. 1. It is immediately apparent from the graphs that the fecal excretion of ingested Co^{60} reached a peak on the second day after dosing, as opposed to the first day after intravenous injection. This observation reflects the fact that most of the fecal Co^{60} following the injected dose probably entered the lower portion of the gastro-intestinal tract and hence was excreted faster than the oral Co^{60} which had to traverse the entire tract. Regarding the percentage composition of Co^{60} in the fecal fractions it can be calculated from Fig. 1 and 2 that over a 7-day period an average of 12% of the fecal Co^{60} from the orally dosed sheep was in the vit. B_{12} fraction, as opposed to 2% after injection. In absolute amounts, then, 10% of the orally administered inorganic Co^{60} appeared in the fecal B_{12} fraction, whereas only 0.2% of the injected dose was found in the feces as vit. B_{12}. These data substantiate the concept that appreciable amounts of vit. B_{12} can be synthesized from ingested cobalt, and since the intravenously injected cobalt apparently entered the alimentary tract below the site of most active B_{12} biosynthesis, it may be assumed most of the biosynthesis of vit. B_{12} occurs above the



PER CENT OF DAILY Co^{60}	1	2	3	4	5	6	7
Total Co^{60}	100	100	100	100	100	100	100
Bound Co^{60}	83.4	79.0	79.0	82.9	80.4	82.4	82.0
Inorganic Co^{60}	15.4	18.2	18.1	14.5	18.0	15.8	16.0
Vit B_{12}-Like Co^{60}	1.2	2.8	2.9	2.6	1.6	1.8	2.0

FIG. 2. Average excretion of Co^{60} in feces of two sheep after intravenous injection plotted as % of dose/day; the tabulated values indicate the % of total Co^{60} represented by each fraction. The total fecal excretion for the week amounted to 8% of the dose.

duodenum, probably in the rumen.

It may be further noted in Fig. 1 that the absolute amount of cobalt-labeled B_{12} excreted in the feces was highest on the fourth day, while the peak excretion of total Co^{60} and the other fractions occurred on the second day. Moreover, the B_{12} fraction increased from 4% of the total Co^{60} excreted on the first day to 20% on the fourth day, after which time it remained essentially constant. These data, when considered in terms of the rate of movement in the tract, give a rough indication of the rate and degree of vit. B_{12} formation in the digestive tract. In Fig. 2, on the other hand, it is seen that after injection the fecal B_{12} fraction was remarkably constant at 2 to 3% of the total fecal Co^{60} except for the first day, when the value was low. Again, this observation probably indicates a time lag due to the formation of vit. B_{12} and subsequent movement down the tract. In this connection it should be noted that the fractional composition of the Co^{60} excreted every day by the injected sheep was very similar to that of the first day's feces from the animals dosed orally. This relationship may be taken to indicate that the ingested Co^{60} reaching the feces on the first day passed through the rumen too quickly to be acted upon appreciably by the organisms present, and hence behaved similarly to the Co^{60} reaching the tract after intravenous injection.

Urinary excretion. Values for the daily excretion of total, B_{12}-like, and inorganic Co^{60} in the urine are presented in Table I. Urine was found not to contain an insoluble bound fraction. In general, the total urinary Co^{60} following both routes of administration was highest on the first day and decreased steadily thereafter. Especially noteworthy was the large amount of the intravenous dose excreted in the urine on the first day, followed by a sharp drop on the second day. The percentage of the dose in both the B_{12} and inorganic fractions followed the same general pattern as the total Co^{60}, but it is important to note that the injected animals were excreting about 5 times as much radioactive vit. B_{12} as those dosed orally. However, as shown in the last 2 columns of Table I, the percentage of total

TABLE I. Urinary Excretion of Total Co^{60}, Inorganic Co^{60} and Vit. B_{12}-Like Co^{60} after Oral and Intravenous Administration of Co^{60} to Sheep.

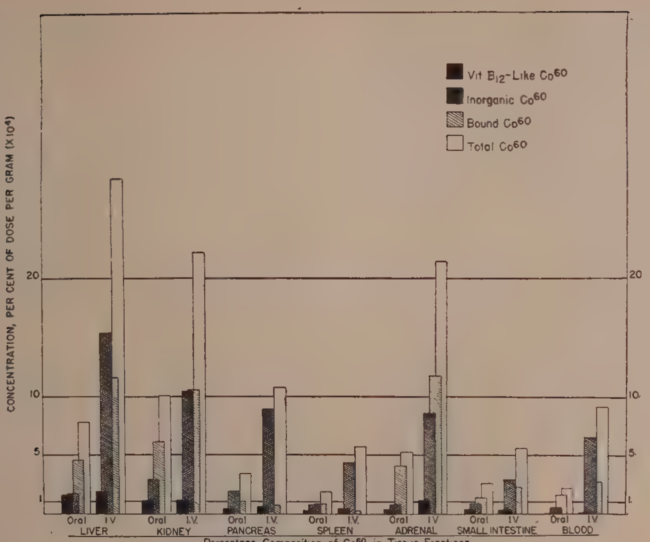
	Total Co^{60}, % of dose		Inorganic Co^{60}, % of dose		Vit. B_{12}-like, % of dose		% of total Co^{60} in urine represented by vit. B_{12}	
Days	Oral	I.V.	Oral	I.V.	Oral	I.V.	Oral	I.V.
1	4	57.3	3.8	55.2	.21	2.1	5.2	3.7
2	3.7	6.3	3.5	5.6	.24	.62	6.5	9.9
3	1.5	6.8	1.4	6.3	.12	.51	8.2	7.6
4	.7	2.3	.6	2	.07	.27	9.9	11.9
5	.3	2.4	.3	2.1	.03	.31	8.7	13
6	.2	1.1	.2	.9	.02	.14	9.5	13.2
7	.4	1.5	.4	1.4	.02	.10	3.9	7
Total	10.8	77.7	10.2	73.5	.71	4.05	7.4*	9.5*

* Averages.

urinary Co^{60} made up by the B_{12} fraction was very nearly the same for both injected and orally dosed sheep, with the values for the injected animals tending to be slightly higher. These percentages differed from the general pattern of total Co^{60} excretion in the urine in that they were low on the first day, increased up to the 6th day, and had started to drop on the 7th day. This behavior probably is another manifestation of the rate of vit. B_{12} formation. Without doubt, the most significant import of these data is concerned with the source of the relatively large amounts of radioactive B_{12} in the urine of the intravenously injected sheep. Since it has been shown that little, if any, vit. B_{12} biosynthesis occurred in the gastrointestinal tract of the injected animals, it seems apparent that the peripheral tissues must have been responsible for some synthesis. The similarity of the values for oral and injected sheep in percentage of total Co^{60} as B_{12} can be interpreted as meaning that the cobalt in the blood and tissues was handled similarly in both cases. Furthermore, the small percentage of the ingested dose excreted as urinary B_{12} indicates that the biosynthesis which has been shown to take place in the tract of the orally dosed animals did not contribute significantly to the amount of vit. B_{12} in the urine, nor presumably to the amount reaching the tissues.

Tissue concentration. Fig. 3 presents values for the concentration of total Co^{60} and of the B_{12}-like, inorganic, and bound fractions in various tissues at the time of sacrifice. Average concentrations for each "oral" tissue are shown side by side with the concentrations of their "i.v." counterparts to facilitate comparison. As in Fig. 1 and 2, values for the percentage composition of Co^{60} in the tissue fractions are tabulated directly below the bar graph. The distribution of total cobalt is in general agreement with findings reported for other species(6-11). Following both modes of administration the highest concentrations of Co^{60} were found in the liver, kidney, adrenal, and pancreas. And, as would be expected from the excretion data, the concentrations of total Co^{60} were higher following an intravenous dose than after oral administration of the same amount of activity. In comparing the concentrations of Co^{60} fractions in the tissues it is noted that the concentration of the bound fraction was generally higher after an oral dose than after injection, whereas the reverse was true concerning inorganic Co^{60}. These observations probably are indications of the source of Co^{60} in each case, since all of the intravenous Co^{60} was injected as inorganic, whereas quite probably much of the ingested Co^{60} was already bound when absorbed. Obvious exceptions to this generalization, however, were the pancreas and spleen, in which there was little bound but much inorganic Co^{60} after either administration route. The adrenal glands, on the other hand, were noteworthy for the relatively high concentration of bound Co^{60} after intravenous injection.

Of particular importance is the fact that a higher tissue concentration of radioactive vit. B_{12} was obtained after intravenous injection of Co^{60} than after oral administration, even



Percentage Composition of Co^{60} in Tissue Fractions

	Liver Oral	Liver I.V.	Kidney Oral	Kidney I.V.	Pancreas Oral	Pancreas I.V.	Spleen Oral	Spleen I.V.	Adrenal Oral	Adrenal I.V.	Small Intestine Oral	Small Intestine I.V.	Blood Oral	Blood I.V.
Total Co^{60}	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Bound Co^{60}	58	40	60	48	33	7	45	6	77	58	53	41	73	29
Inorganic Co^{60}	22	53	29	47	55	87	42	85	15	40	32	53	25	70
Vit B_{12}-Like Co^{60}	20	7	11	5	12	6	13	9	8	5	15	6	2.3	0.8

FIG. 3. Average concentrations of Co^{60} in different chemical fractions of various tissues of sheep after oral and intravenous administration. The tabulated values indicate % of total Co^{60} represented by each fraction.

though the vit. B_{12} fraction represented a smaller percentage of the total Co^{60} present in the former instance. Thus these data confirm decisively what the excretion data suggest, that some internal biosynthesis of vit. B_{12} must have taken place. The possibility that Co^{60} entered the B_{12} molecule by exchange seems unlikely in view of the failure of extensive efforts of Fantes *et al.*(17) to label B_{12} *in vitro* by exchange. Also, Chow *et al.*(14) found that no inorganic Co^{60} could be recovered in the urine of rats fed radioactive vit. B_{12}, indicating that no exchange took place *in vivo* under their conditions.

Indications regarding possible sites of B_{12} biosynthesis in the peripheral tissues may be obtained by presentation of the data in Fig. 3 in another way. A measure of the enrichment, or the degree of the accumulation, in a tissue may be obtained by calculating the ratio of the tissue B_{12}-Co^{60} to the blood B_{12}-Co^{60}. These ratios for several tissues are presented in the first 2 columns of Table II. When vit. B_{12} is introduced into the circulating blood one would expect a similar distribution pattern whether the B_{12} were introduced by direct injection or absorption from the tract. On the other hand, if vit. B_{12} were formed within a tissue from inorganic cobalt we might presume that this tissue would show a greater enrichment of the vitamin after injection than after oral administration of inorganic cobalt. By comparing the "i.v." enrichment against the "oral" enrichment after Co^{60} administration (column 3 of Table II), it is noted that only in the case of the adrenals and possibly the spleen was there a significantly greater enrichment of B_{12}-Co^{60} from the intravenously administered Co^{60}. This is considered to be presumptive evidence of vit. B_{12} biosynthesis in these tissues. As postulated, the ratios for the other tissues approach 1. The low value for the small intestine may be explained by the fact that in the orally dosed animals this tissue was continuously exposed to a considerable amount of B_{12} that had been synthesized in the tract.

The tissues were less efficient than the micro-organisms of the digestive tract in the biosynthesis of vit. B_{12} as can be shown by estimating the total amount of B_{12} formed in each case. On the basis of the B_{12}

TABLE II. Tissue Accumulation of Vit. B_{12}-Co^{60} from Blood after Oral and Intravenous Administration of Co^{60} to Sheep.

Tissue	Conc. of Vit. B_{12}-Co^{60} in tissue / Conc. of Vit. B_{12}-Co^{60} in blood		I.V.* / Oral
	Oral	I.V.	
Liver	31.2	27	.87
Kidney	21.4	16.6	.78
Pancreas	8.2	9.6	1.17
Spleen	5	7	1.40
Adrenals	8.2	16.4	2
Small intestine	7.6	4.4	.58
Bone marrow	1.2	1.1	.92

* This represents the value in column 2 divided by that in column 1 (see text for explanation).

concentrations shown in Fig. 3, the average carcass concentration of B_{12} for the injected animals might be estimated liberally as 5 x 10^{-5}% of the dose per g. The total amount of B_{12} formed would then be (5 x 10^{-5}) (36,000 g body weight) = 1.5% of the dose, as compared with 10% of the dose found in the feces B_{12} of the orally dosed animals. It appears that at least 10 times as much of an oral dose of inorganic Co^{60} was converted to vit. B_{12} as was the case after intravenous injection. This is a conservative estimate and the true difference is probably much wider inasmuch as a liberal tissue value was used and the feces value is probably low, since no correction was made for degradation of the B_{12} in the tract. Barbee and Johnson(13) have shown that considerable degradation of vit. B_{12} occurs in the gastrointestinal tract of the rat.

Discussion. It is generally considered that cobalt is functional in all species in connection with its direct hematopoietic action. In ruminants there is an additional requirement which is concerned with the external function of cobalt in the rumen contents(18,19), which is probably related to bacterial action(20,21) and the production of B-vitamins(22,23). Becker and Smith(24) have shown that 1 mg of cobalt fed orally to cobalt deficient lambs will alleviate deficiency symptoms, whereas 1 mg of cobalt administered subcutaneously will not. On the basis of our results the tissues of their injected animals must have contained as much if not more vit. B_{12} than those of the orally supplemented lambs. This can only be interpreted to mean that either the vit. B_{12} as characterized in this paper was physiologically ineffective, at least insofar as gross symptoms of cobalt deficiency were concerned, or that the rumen requirements were not satisfied. Smith *et al.*(23) have recently reported that injection of 0.15 mg of a vit. B_{12} preparation produced a response in cobalt-deficient lambs. This would seem to indicate that the vit. B_{12} synthesized in the tissues from parenterally administered cobalt was physiologically ineffective, in contrast to that which reached the tissues after synthesis in the rumen.

Summary. 1. Excretions and tissues from wether lambs dosed orally and intravenously with inorganic cobalt-60 were measured for total radioactivity and also for radioactivity in fractions characterized chemically as vit. B_{12}-like, inorganic and bound. 2. After oral administration there was considerable biosynthesis of vit. B_{12} in the tract, probably occurring mainly in the rumen. After intravenous administration there was only slight, if any, biosynthesis in the tract, but considerable formation of vit. B_{12} in the tissues with the suggestion that the adrenals and spleen were mainly responsible. Overall, there was more than 10 times as much Co^{60} converted to B_{12} after ingestion than after intravenous administration. 3. The concentrations of vit. B_{12}-like Co^{60} in the tissues after intravenous injection were slightly higher than after ingestion of equal amounts of Co^{60}. 4. In the light of results on the response of cobalt-deficient lambs to parenterally administered vit. B_{12}, it is suggested that the vit. B_{12}-like material synthesized by the tissues from inorganic cobalt is physiologically ineffective in relieving cobalt-deficiency symptoms in sheep.

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Viability of the Lansing Strain of Poliomyelitis Virus in the Bat (*Eptesicus fuscus*). (19587)

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The present study was undertaken in order to determine whether the bat (*Eptesicus fuscus*) might be a reservoir for poliomyelitis virus. Since under normal conditions the most probable method of exposure for the bat would be by the oral or intranasal routes, these 2 methods were used in the following experiment. As a control on the susceptibility of the bats, they were also inoculated by the intracerebral route.

Materials and methods. The Lansing strain of poliomyelitis virus was used. The strain was originally obtained by this laboratory in the form of mouse-brain suspension of the 200th mouse passage from Dr. David Bodian of the Poliomyelitis Research Center, Johns Hopkins University, Baltimore, Md. The virus had been passed 5 additional times in mice and once in cotton rats at the initiation of the present study. A 10% virus-bearing cotton-rat brain suspension was used as the inoculum for all bats. According to the Reed-Muench method of calculation(1) this material titrated $10^{-4.5}$ in cotton rats by intracerebral inoculation. The bats used in the present experiment were of the species *Eptesicus fuscus* and were in hibernation when they were obtained. They were kept kept in a heated room at the laboratory for 10 days before inoculation. During this period, all weak or inactive bats were discarded. The bats were divided into 3 groups of 28 bats each, and each group was exposed to the virus by the oral, intranasal, or intracerebral route. Bats of the intranasal and intracerebral groups were lightly anesthetized with ether before administration of the virus. The inoculum for each group was as follows: intracerebral, 0.03 ml per bat; intranasal, 0.02 ml per bat; and oral, 0.1 ml per bat. Following exposure to the virus, the bats of different groups were kept in separate cages in an isolation room with a lower temperature. Most of the bats went into hibernation and the remainder were very lethargic. On the 5th day post inoculation, 6 bats from each group were sacrificed and the following pools of tissues and fecal material made from bats of the same group: brains and spinal cords, hearts, lungs and tracheas, spleens, livers, kidneys and bladders, and intestines and feces. Aseptic technics were maintained and different instruments were used in removing each type of tissue. These 21 pools were stored in a —40°C deep freeze cabinet. On the 12th, 20th, and 30th days after inoculation the same procedure was repeated using 5 bats from each group. Similar pools of tissues were made from one bat of the oral group which died 18 days after inoculation. The bats which remained after the 30-day period were discarded. The pools of tissue were removed from the deep freeze, thawed, ground with alundum, and diluted in as small an amount of physiological saline as possible. The suspensions were centrifuged at 1500 r.p.m. for 2 minutes. The sediment was discarded and the supernatant was treated with penicillin and streptomycin in order to eliminate any possible bacterial contamination.

The eastern cotton rat (*Sigmodon hispidus hispidus*) rather than the mouse was chosen as the test animal because of its higher susceptibility to Lansing strain poliomyelitis. Four hundred fifty-five cotton rats were divided into 91 groups of 5 rats each. Each of the 5 rats from one group were inoculated intracerebrally with 0.06 ml of one of the suspensions described above. These rats were observed twice daily for 18 days. Rats dying within the first 48 hours were discarded. In cases where 3 or more rats died within this period, more rats were injected so that a minimum of 3 rats survived after 48 hours. The brains of rats evidencing symptoms of central nervous system (CNS) involvement or dying after the first 48 hours were removed aseptically. Histopathological examinations were made on sections of each of these rat brains by Dr. Theodore Winship and Dr. David Bell of Garfield Memorial Hospital, Washington, D.C. One-half of each brain was stored in 50% glycerine until the end of the 18-day observation period. They were then ground separately with alundum and diluted to 20% suspensions with physiological saline. Each of 5 rats was injected intracerebrally with 0.06 ml of one of these suspensions. Those rats which died within the first 48 hours were discarded, and the remainder were held for an 18-day observation period.

Results. None of the bats inoculated showed any evidence of CNS involvement following inoculation. The one bat from the oral group which died on the 18th day after exposure was found dead in the morning and no symptoms had been noted the previous day.

Table I shows that 9 rats became sick or died following injection with the bat-brain suspensions. The 3 columns under the heading "Inoculum" indicate the bat material from which the inoculum was prepared. Of the 9 rats succumbing, 4 were injected with suspensions of feces and intestines, 3 with suspensions of brains and cords, and only one each with a suspension of hearts and of kidneys and bladders. A considerable number of rats inoculated with the pools of kidneys and bladders died within the first 24 hours. This high mortality rate might have been due to a toxic factor in the suspensions.

Histopathological examinations of the rat brain sections showed no specific changes that might be due to infectious agents. The predominant feature in all cases was capillary dilatation and engorgement with blood. No perivascular cuffing or degenerative changes in the neuronal or glial elements were seen.

Attempts to pass the lethal agent were unsuccessful. Rats of the 2nd passage remained normal during the 18-day observation period.

Discussion. The fact that the bats were in hibernation during the period following exposure to the virus made it rather difficult to determine symptoms of illness. However, no symptoms of CNS involvement such as paralysis or tremors were noted.

In the 4 cases where the rats succumbed following inoculation with suspensions of bat feces and intestines, the inoculum was still contaminated with bacteria in spite of the treatment with penicillin and streptomycin. It is therefore possible that the symptoms and deaths were due to a bacterial infection. However, although symptoms of CNS involvement were present in the rats succumbing on the 11th and 12th days after inoculation, no evidence of a bacterial encephalitis or menin-

TABLE I. Rats Dying or Showing CNS Involvement Following Injection with Bat-Brain Suspension. One rat in each experiment.

Inoculum				
Bat group	Days after inj. tissues were harvested	Pool	Incubation, days	Symptoms or death
Oral	5	Feces and intestines	11	Paralysis hind legs
	12	” ” ”	12	Slight tremors; partial paralysis
	12	Hearts	6	Paralysis
	30	Brains and cords	8	Dead*
	30	Kidneys and bladders	4	Paralysis hind legs
Intranasal	12	Feces and intestines	6	Dead*
	30	Brains and cords	7	” *
Intracerebral	5	” ” ”	8	” *
	12	Feces and intestines	6	” *

* These rats were dead when examined in the morning although they had shown no symptoms the previous afternoon.

gitis were noted on histopathological examinations nor could the agent be passed serially to more rats. Furthermore, the pathological findings in all 4 cases were almost identical with those in the other 5 rats which succumbed following injection with bacteria-free material.

Summary. Different groups of bats (*Eptesicus fuscus*) were exposed to the Lansing strain of poliomyelitis virus by the oral, intranasal, and intracerebral routes. Attempts were made to isolate the virus in cotton rats from suspensions of various tissues and feces of bats sacrificed on the 5th, 12th, 20th, and 30th days after exposure. Nine of the rats died or were paralyzed on the first passage. Four of these rats had been injected with bat feces and intestines, 3 with bat brains and cords, one with bat hearts, and one with bat kidneys and bladders. However, histopathological examinations showed no typical lesions of poliomyelitis, and attempts to produce symptoms in rats of the 2nd passage were unsuccessful.

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Effects of Isonicotinic Acid Derivatives on Tubercle Bacilli.* (19588)

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The mechanism underlying the action of synthetic chemicals and antibiotics on bacteria remains a debated question. The possibility that the tuberculocidal or tuberculostatic effect of some chemicals and antibiotics may be due to their action on the respiratory enzymes of tubercle bacilli has long been under investigation in this laboratory.

The effect of isonicotinic acid hydrazide and 1-isonicotinyl-2-isopropylhydrazine on oxygen consumption, succinic dehydrogenase and catalase activity of virulent, streptomycin-sensitive and streptomycin-resistant strains of human type tubercle bacilli, as well as on the attenuated, streptomycin-sensitive, bovine type tubercle bacilli BCG, was studied.

Material and methods. Cultures of tubercle bacilli were grown on Sautons synthetic medium for 7 to 19 days, during which time they produced a heavy wrinkled film on the surface of the medium while the underlying culture medium remained clear. The bacilli

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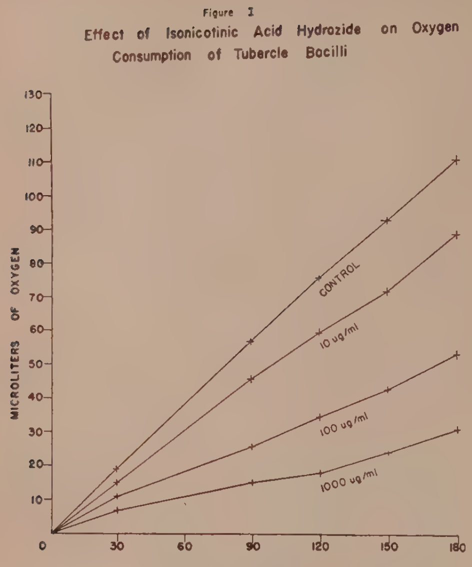

FIG. 1.

were collected on a sintered glass filter of coarse porosity, where they were washed several times with isotonic saline solution buffered to pH 7.0. The washed bacillary mass was weighed, ground to a uniform paste with glass beads and suspended in buffered saline to yield a concentration of 50 mg moist weight per ml. Oxygen consumption was determined by the usual Warburg technic. One ml of the bacillary suspension was transferred to Warburg flasks and one ml of a solution of the isonicotinic acid hydrazide or 1-isonicotinyl-2-isopropylhydrazine, made up to the desired concentration with buffered saline, was added. The mixture was permitted to equilibrate for 30 minutes at 37°C, after which time readings were recorded at 30 minute intervals for a period from 2 to 3 hours.

Results. Unlike the chemicals and antibiotics studied previously, under the same conditions, isonicotinic acid hydrazide significantly reduced the oxygen consumption of tubercle bacilli. The per cent reduction of oxygen consumption paralleled the concentration of isonicotinic acid hydrazide and bore no relationship to the sensitivity or resistance of the organisms to streptomycin. The results of a typical experiment with a virulent human type streptomycin-sensitive tubercle bacillus are presented in Fig. 1.

The oxygen consumption of the avirulent BCG culture was inhibited to a less degree by isonicotinic acid hydrazide than was that of the virulent strains of tubercle bacilli used in the experiments here recorded. The percent inhibition of oxygen consumption for both virulent and attenuated strains of tubercle bacilli is presented in Table I. The inhibitory action of isonicotinic acid hydrazide on oxygen consumption was most marked on 6- to 12-day-old cultures of tubercle bacilli and significantly less marked on 12- to 19-day-old cultures. The cultures of tubercle bacilli used in this study were inhibited by a concentration of 0.15 μg/ml of isonicotinic acid hydrazide in Youmans medium containing 0.5% fraction V bovine albumen.

Catalase. The effect of isonicotinic acid hydrazide on the enzymatic activity of catalase of virulent and attenuated strains of tubercle bacilli was determined according to the method of Finlayson and Edson(1). One ml of a bacillary suspension containing 0.75 mg moist weight of the virulent organisms was transferred to Warburg flasks while 2.5 mg in 1 ml of 0.015 M phosphate buffer, pH 7.4 of the avirulent BCG culture was transferred to the flasks. The larger amount of the attenuated BCG culture was used, because unpublished studies indicate that the catalase activity of attenuated strains of tubercle bacilli is significantly less than that of virulent strains. To the bacillary suspension there was then added isonicotinic acid hydrazide to give a final concentration of either 10, 100 or 1000 μg/ml. After 30 minutes equilibration at 37°C, 0.3 ml of a 0.05 M solution of hydrogen peroxide in 0.005 M phosphate buffer, pH 7.4, was tipped into the flask from the side arm. Manometric readings of the oxygen evolved were taken at 10-minute intervals for 30 minutes. As a control, distilled water was substituted for the isonicotinic acid hydrazide. The results obtained are presented in Table I.

It will be noted from Table I that while isonicotinic acid hydrazide inhibited the oxygen consumption of the virulent strain of tubercle bacilli to a greater degree than the attenuated strain, no such striking reduction

TABLE I. Effect of Isonicotinic Acid Hydrazide on Oxygen Consumption and Catalase Activity of Virulent and Attenuated Cultures of Tubercle Bacilli.

μg/ml isonicotinic acid hydrazide	% reduction of oxygen consumption		% reduction of catalase activity	
	Strains of tubercle bacilli			
	Virulent	Attenuated	Virulent	Attenuated
10	9	4	4	14
100	53	12	28	36
1000	73	27	87	80

TABLE II. Percentage Inhibition of Oxygen Consumption and Catalase Activity of Virulent Tubercle Bacilli by Isonicotinic Acid Derivatives.

Isonicotinic acid derivatives	% reduction of oxygen consumption			% reduction of catalase activity		
	10 μg/ml	100 μg/ml	1000 μg/ml	10 μg/ml	100 μg/ml	1000 μg/ml
Isonicotinic acid hydrazide	9	53	73	4	28	87
1-isonicotinyl-2-isopropylhydrazine	0	0	12	0	0	0

was evident in the relationship of catalase activity to virulence of the bacilli.

Succinic dehydrogenase. The effect of isonicotinic acid hydrazide on this enzyme was investigated by a modification of the procedure described by Kun and Abood(2). To 1 ml containing 75 mg of a bacillary suspension prepared as described above there was added 1 ml isonicotinic acid hydrazide in M/50 sodium succinate to give a final concentration of 1000 μg/ml. This mixture was incubated for one hour, after which time there was added 1.5 mg of neotetrazolium chloride dissolved in M/15 phosphate buffer pH 7.6. After 3 hours incubation at 37°C the neotetrazolium chloride was reduced to a deep red water-insoluble formazan. At the end of this time the reaction was stopped by the addition of 0.5 ml of a 40% solution of trichloracetic acid. The formazan was extracted by vigorous shaking for 30 seconds with 10 ml of ethyl acetate, after which time the mixture was centrifuged. Five ml of the supernatant fluid was transferred to a Klett tube and read in a Klett-Summerson colorimeter. The results were compared with a control tube to which no isonicotinic acid hydrazide had been added. It was found that under these conditions the succinic dehydrogenase activity was not affected by the amount of isonicotinic acid hydrazide used.

Action of 1-isonicotinyl-2-isopropylhydrazine on tubercle bacilli. The action of 1-isonicotinyl-2-isopropylhydrazine on the oxygen consumption, catalase and succinic dehydrogenase activity of tubercle bacilli was determined in the same manner as described above. The growth of the cultures used in these studies was inhibited by 15.0 μg/ml of 1-isonicotinyl-2-isopropylhydrazine in Youmans medium containing 0.5% fraction V albumen. It was found that 100 or 1000 μg/ml of the 1-isonicotinyl-2-isopropylhydrazine slightly inhibited the oxygen consumption of virulent strains of tubercle bacilli as well as the attenuated BCG culture. The catalase and succinic dehydrogenase activity of the virulent strains was not affected by the 1-isonicotinyl-2-isopropylhydrazine. On the other hand, the catalase activity of the attenuated BCG strain was reduced 10, 19 and 25% by 10, 100 and 1000 μg/ml of the compound respectively while the action of succinic dehydrogenase was not affected. The comparative action of isonicotinic acid hydrazide and 1-isonicotinyl-2-isopropylhydrazine on the oxygen consumption and catalase activity of strains of virulent tubercle bacilli is presented in Table II.

It will be observed from this table that comparable amounts of isonicotinic acid hydrazide and 1-isonicotinyl-2-isopropylhydrazine show significant differences in their inhibitory action on oxygen uptake and catalase activity. However, when the inhibitory action of these 2 compounds on the growth of tubercle bacilli is considered as being in the ratio of 100 to 1, it then becomes evident that 10 μg/ml of isonicotinic acid hydrazide has the same effect on oxygen uptake as has 1000 μg/ml of 1-isonicotinyl-2-isopropylhydrazine.

Conclusions. 1. The oxygen consumption and catalase activity of both virulent and attenuated strains of tubercle bacilli was reduced significantly by isonicotinic acid hydrazide from 2 different laboratories while the activity of succinic dehydrogenase was unaffected. 2. 1-isonicotinyl-2-isopropylhydrazine slightly inhibited the oxygen consumption of both virulent and attenuated strains of tubercle bacilli, but did not modify the action of succinic dehydrogenase of these cultures. However, the catalase activity of the attenuated BCG strain was significantly reduced, while the catalase activity of the virulent strains was unaffected.

We are indebted to Hoffmann-La Roche, Nutley, N. J., for samples of Isonicotinic Acid Hydrazide and 1-isonicotinyl-2-isopropylhydrazine and to E. R. Squibb & Sons, New Brunswick, N. J. for Isonicotinic Acid Hydrazide.

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Effect of Age on Wallerian Degeneration in the Rat.* (19589)

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If a peripheral nerve is cut that portion of the nerve distal to the point of section undergoes a series of changes characteristic of Wallerian degeneration. Previously it has been shown that in cat sciatic nerve degenerating after nerve section there is an increase in the concentration of nucleic acid (1) related to cellular proliferation, and a decrease in the concentration of phospholipid (2) related to the destruction of the myelin sheath. Similar changes occur in the sciatic nerve of the rat, although the degeneration process is speeded up considerably(3). In experiments preliminary to those described in the above paper it became apparent that in the sciatic nerve of the rat the concentration of both nucleic acid and phospholipid varied with the age of the animal. Since it has been frequently stated that Wallerian degeneration proceeds more rapidly in younger animals(4), it was decided to study the effect of age on the concentration of nucleic acid and phospholipid both in normal nerves and in the peripheral stump of nerves degenerating after nerve section.

Methods. The sciatic nerve on one side of each of 2 groups of male Sprague-Dawley rats was sectioned high in the thigh. One group of rats was 60 days old (body weight = 205 ± 5 g) and the other was 160 days old (body weight = 419 ± 8 g). Both groups were maintained on an adequate synthetic diet. After either 8 or 16 days the animals were killed and the distal degenerating segment of nerve removed. In none of the animals was there postmortem evidence of regeneration. A similar length of sciatic nerve was taken from the opposite side to serve as a control. Phospholipid was determined in a lipid extract of the nerve(2). Nucleic acid was determined by ultraviolet absorption using a hot trichloroacetic acid extract of the residue remaining after the extraction of lipids and acid-soluble compounds(1). Details of the operation, analytical procedures, and the method of recording the results have already been published(3).

Results. Nucleic Acid. Table I shows that the concentration of nucleic acid in the sciatic nerves of the 60-day rats was significantly ($P < 0.001$) greater than that in the nerves

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TABLE I. Effect of Age on Concentration of Nucleic Acid in Sciatic Nerve of the Rat. (mg P/100 g wet wt.)

		60-day group	160-day group	Mean difference	P*
Intact nerve	Mean	19.3± .57† (16)‡	11.7± .21† (16)‡	7.6± .61†	<.001
8 days after section	Mean	51.8±2.59 (8)	36.7± .96 (5)	15.1±2.76	<.001
	Mean increase (0-8 days)	32.5±2.65	25 ± .98	7.5±2.83	<.01
16 days after section	Mean	56.3±2.66 (8)	48.8±1.66 (10)	7.5±3.14	<.05
	Mean increase (8-16 days)	4.5±3.71	12.1±1.92	−7.6±4.18	>.05
	Mean increase (0-16 days)	37 ±2.72	37.1±1.67	− .1±3.19	>.9

* Significance of mean difference between 60-day group and 160-day group by 't' test.
† Standard error of mean. ‡ No. of animals in group.

TABLE II. Effect of Age on Concentration of Phospholipid in Sciatic Nerve of the Rat. (mg/100 mg wet wt.)

		60-day group	160-day group	Mean difference	P*
Intact nerve	Mean	8.14±.17† (16)‡	7.66±.13† (17)‡	−.48±.21†	<.05
8 days after section	Mean	4.90±.30 (8)	6.48±.25 (6)	1.58±.39	<.01
	Mean decrease (0-8 days)	3.24±.35	1.18±.28	−2.06±.45	<.001
16 days after section	Mean	2.97±.19 (8)	3.43±.11 (10)	.46±.22	>.05
	Mean decrease (8-16 days)	1.93±.36	3.05±.27	1.12±.45	<.02
	Mean decrease (0-16 days)	5.17±.26	4.23±.17	−.94±.31	<.01

* Significance of mean difference between 60-day group and 160-day group by 't' test.
† Standard error of mean. ‡ No. of animals in group.

of the 160-day animals. As found previously for both the cat(1) and the rat(3), there was an increase in the concentration of nucleic acid during the period 0-8 days after nerve section. The table also shows that for this period the mean increase for the 60-day animals was significantly ($P < 0.01$) greater than that for the 160-day animals. It will be noted that the increase in the concentration of nucleic acid over the 0-16 days period was not significantly different in the two groups. The nucleic acid entered the nerve more rapidly in the younger animals.

Phospholipid. Table II shows that, as for nucleic acid, the concentration of phospholipid was significantly ($P < 0.05$) greater in the nerves of the 60-day rats than in the nerves of the 160-day animals. In confirmation of previous findings(3), there was a decrease in the concentration of phospholipid during both the periods 0-8 days and 8-16 days after nerve section. Table II shows that during the period 0-8 days the mean decrease for the 60-day animals was considerably ($P < 0.001$) greater than that for the 160-day group, whereas during the period 8-16 days the decrease for the 160-day animals was greater than that for the 60-day group ($P < 0.02$).

Discussion. These experiments show that the concentration of both nucleic acid and phospholipid in the sciatic nerve of the rat is greater in younger animals than in older. After nerve section the more rapid increase in the concentration of nucleic acid in the younger animals probably represents a more rapid cellular proliferation, and the more rapid decrease in the concentration of phospholipid probably represents a more rapid loss of myelin material. This confirms previous histological studies(4) showing that Wallerian degeneration proceeds more rapidly in younger animals.

In the younger animals the more rapid increase in the concentration of nucleic acid is accompanied by a more rapid decrease in the concentration of phospholipid. This finding is consistent with the hypothesis that the proliferating cells provide enzymes for the chemical destruction of the lipids of the myelin sheath. Results similar to those reported above have been obtained with other groups of animals kept under a wide variety of dietary conditions.

Summary. The sciatic nerves of a group of rats 60 days old contained a higher concentration of both nucleic acid and phospholipid than the nerves of similar rats 160 days old. After section of the nerve there was an increase in the concentration of nucleic acid and a decrease in the concentration of phospholipid in both groups. These changes occurred more rapidly in the younger animals.

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Effect of Estrogens on Serum Lipids and Low Density Lipoproteins in Men and Women.[*] (19590)

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Gofman *et al.*(1) have demonstrated significant sex differences in the concentration of the Sf 12-20 beta-lipoproteins, in that women (25-40 years of age) showed about 25% less of these lipoproteins in their blood than men of similar age group. Since these lower values correlate well with the lower incidence of atherosclerosis in young women it seemed important to observe the effect of administering the sex steroid hormones on the serum lipids and lipoproteins in both sexes. Such observation might help to explain the observed sex differences in atherosclerosis. Eilert(2) did report that estrogens induced a sharp reduction in the ratio of total serum cholesterol to phospholipid because of a rise in phospholipid and a fall in total cholesterol. These findings are contrary to the observations being reported in this study.

Our clinical observations were derived from the study of 16 males and 15 females (whose ages range from 55-65 years) who were given estradiol[†] in variable physiologic doses of 0.25-0.75 mg daily for periods of 2-4 months. Blood specimens were taken before and at monthly intervals after the start of estrogen administration for the determination of the total serum lipids, cholesterol, cholesterol esters, phospholipids, cholesterol/phospholipid ratio, as well as the serum low density lipoproteins.[‡]

TABLE I. Average Values of Blood Lipids in Males and Females after Estrogen Therapy.

	Cholest.	Phospho-lipids	Ratio C/P*	Sf 10-20
		Females		
Before therapy	285	12.9	22.4	51
1 mo	290	12.7	23.1	52
2	282	11.4	24.7	55
3	282	12.6	22.4	51.4
		Males		
Before therapy	248	10.9	23	55.2
1 mo	244	9.7	25.1	49.4
2	270	11.1	24.2	52.8
3	266	10.5	25.3	54.7

* Ratio of cholesterol to phospholipids.

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[†] Supplied by the Schering Corp., Bloomfield, N. J.

[‡] All ultracentrifugal determinations were performed at the Donner Laboratory, University of California, Berkeley.

TABLE II. Effect of Estradiol, .75 mg Daily, in a 64-Year-Old Male.

	Sf 10-20	Cholest.	Cholest. esters, %	Phospholipids	Ratio C/P*	Total lipids
1/25	43	201	73	8.1	24.9	610
	Medication—started					
2/15	66	178	76	8.3	21.4	550
3/1	36	219	74.5	8.7	25.2	470
3/29	57	235	70	9.7	24.2	510
5/5	59	265	68	10.1	26.2	520
	Medication—stopped					
6/7	55	210	75	9.5	22.1	480

* Ratio of cholesterol to phospholipids.

TABLE III. Effect of 5 mg Estradiol Dipropionate I.M. in a 25-Year-Old Female Castrate.

	Sf 10-20	Cholest.	Phospholipids	Ratio C/P*	Total lipids
2/12	36 (inj.)	287	12.4	23.1	700
14	49	260	14.2	18.3	680
16	41	304	12.4	24.5	610
20	29	280	15.4	18.2	630

* Ratio of cholesterol to phospholipids.

To test the influence of larger doses of estrogen on these lipid factors 5.0 mg of estradiol was injected in 4 subjects, one of whom was a young female castrate (Table III).

No dietary nor any other medical management was changed during these experimental trials.

The data obtained are summarized in Table I. Table II shows the values obtained in a 64-year-old man in whom the doses used caused tenderness and hypertrophy of the breasts.

It is readily apparent that the administration of estrogen is followed by no marked nor sustained change in any of the serum lipid factors nor in the cholesterol-phospholipid ratio, nor did analysis of the Sf 12-20 lipoproteins show any consistent trend.

In our hands no evidence has been obtained to indicate that estradiol in either physiological or pharmacological doses influences the serum lipids or the low density lipoproteins.

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Ineffectiveness of 688A (N-Phenoxyisopropyl-N-Benzyl-β-Chloroethylamine Hydrochloride) in Treatment of Essential Hypertension. (19591)

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(Introduced by Irvine H. Page.)

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Certain chloroethylamine derivatives can in varying degree block adrenergic and sympathetic activities. Thus, some patients with essential and malignant hypertension respond to intravenous injection of Dibenamine by more or less prolonged decreases of arterial pressures(1,2). Availability of an orally active(3) drug of this type, 688A prompted observations of its effectiveness in the prolonged treatment of essential hypertension. As this study was completed, Haimovici, Moser and Krakauer(4) reported preliminary studies which indicated that 688A was therapeutically effective in essential hypertension.

Methods. This report is based on data from 8 of 25 patients treated; some observations are omitted or briefly noted because a) the drug was sometimes used in the terminal phases of malignant hypertension (6 patients), b) concurrently with another drug (with

TABLE I. Summary of Effects on Average Arterial Pressure of Administration of 688A in 8 Patients with Essential Hypertension. Dosage indicated is the daily maximum administered and/or tolerated. Arterial pressures are means of control and treatment observations. Differences are indicated as significant (+) when difference between means is greater than 3 times and standard error of the difference.

		Means of arterial pressures, mm Hg			Significance of diff. between	
		Control	Treatment			
Treatment, wk	Dose, mg	Supine 1	Supine 2	Standing 3	2 and 1 S/D	3 and 1 S/D
25	560	165/116	164/120	148/115	0/0	+/0
21	80	185/121	171/108	140/ 97	0/+	+/+
9	520	200/153	184/142	190/149	0/0	0/0
3	1200	204/130	220/140	169/113	0/0	+/+
4	40	154/112	144/103	147/108	0/0	0/0
26	40	198/110	179/101	172/ 94	+/+	+/+
3	160	197/117	178/113	181/112	+/0	+/0
2	240	187/120	185/122	168/120	0/0	+/0

Veriloid twice and once with sodium nitroprusside), c) the follow-up was inadequate (7 patients) or very brief. The ages of the 8 patients who suffered from moderately severe or severe essential hypertension ranged from 20 to 60 years. Dosages and duration of treatment appear in Table I. Treatment usually began with the administration of 20 mg twice daily, increased to 4 times daily after a day or two and subsequently adjusted as tolerated. Nasal stuffiness, Horner's syndrome and orthostatic hypotension were taken as criteria of adequate medication; all these tended to remit as treatment was prolonged. *Control observations* consisted in evaluation of the patients' cardiovascular-renal status by methods currently used in the Research Division; 7 of the 8 patients were hospitalized for 2 weeks or more until averages of arterial pressures measured twice daily showed a minimum of variability; in the remaining patient, arterial pressures had been measured at frequent office calls over a period of nearly 2 years. During treatment, pressures continued to be measured twice daily in the supine position and again 3 to 5 minutes after quiet standing. Means of arterial pressures, standard deviations and standard error of differences between means of control supine, treatment supine and treatment orthostatic pressures were calculated by standard methods(5).

A more detailed study of vascular responsiveness carried out by Dr. James McCubbin in one patient is presented in detail below.

Results. 1) *Effect on arterial pressure.* Observations are summarized in Table I. Orthostatic decreases of systolic and diastolic pressures were observed in 3 patients and of systolic pressure only in another 3. Supine systolic and diastolic pressures showed statistically significant, but clinically unimpressive, decreases in 1 patient; 1 patient showed some decrease in supine systolic pressure only and another a decrease in supine diastolic pressure.

2) *Clinical evaluation.* No significant change was observed in the clinical status of any patient.

3) *Side-effects.* The side-effects of which these patients complained were, a) nasal congestion (4 patients), b) giddiness on quiet standing (4 patients), c) increased fatigability (3 patients), d) palpitation on standing (2 patients) and e) "swollen eyes" (1 patient). Epistaxis was not observed in this group, but nasal spotting occurred in some of the patients who were given the drug during the terminal phase of malignant hypertension. The one patient noted above as showing decreases in systolic and diastolic arterial pressures during treatment was especially susceptible to these effects; any increase in dosage over 40 mg daily resulted in disabling orthostatic palpitation and giddiness.

4) *Vascular reactivity.* (patient 4, Table I). This patient's intra-arterial pressure responses to intravenous test doses of adrenaline, noradrenaline and tetraethylammonium chloride (TEAC) were recorded from a Technitrol capacitance manometer. Relevant data are summarized in Table II. Pretreatment tests demonstrated repetitive, marked depressor re-

TABLE II. Vascular Reactivity to Test (Intravenous) Doses of Adrenaline, Noradrenaline and Repetitive Dosage (Sequentially Numbered) of TEAC in a Patient before and at the 7th Day of Treatment with 688A. Responses on 28th day of treatment correspond well with those found in the control period. The stated arterial pressures are levels (systolic/diastolic) measured at the time of the inj.; responses to inj. as indicated as maximum change from this level in mm Hg and decreases in pressure are preceded by a minus (–) sign.

Test drug	Arterial pressure, mm Hg	Response to drug, mm Hg
I. Observations in control period		
Adrenaline	220/118	35/20
		40/25
		20/2
Noradrenaline	230/122	45/12
TEAC 1	265/140	–95/40
2	250/148	–65/88
3	242/145	–58/35
4	230/138	–38/28
5	240/145	–55/85
6	240/148	–48/28
II. Observations on 7th day of treatment		
Adrenaline	230/125	–80/40
Noradrenaline	225/125	5/0
TEAC 1	225/118	15/20
2	220/121	15/24

sponses to TEAC, analogous to those found in dogs with experimental neurogenic (sinoaortic nerve section) hypertension (Page and McCubbin)(6). These animals respond to sympathectomy by a decrease of arterial pressure(7) and demonstrate moderate decreases of arterial pressure during treatment with 688A (McCubbin, unpublished observations). We therefore hoped that this patient might show a sustained favorable effect of treatment. Eighty mg was given 4 times daily on the first day and 120 mg, 4 times daily thereafter. Supine arterial pressure was not affected, although orthostatic hypertension, nasal congestion and Horner's syndrome supervened. Responses to test drugs were redetermined at the end of 1 week. Presence of adrenergic blockade was shown by pure depressor responses to adrenaline and by a damped pressor response to noradrenaline; sympathetic blockade was considered to be present because the responses to TEAC, systematically depressor before treatment, were now repetitively pressor. The dose of the drug was then increased within the limits of tolerance (gauged subjectively and from orthostatic hypertension) to 1200 mg at the end of the third week after discharge. During this time, systolic and diastolic supine arterial pressures had persisted at about control levels. Tests of pressor responsiveness to adrenaline, noradrenaline and TEAC on the 28th day of treatment demonstrated neither adrenergic nor sympathetic blockade.

Discussion. Our observations contrast with those of Haimovici, Moser and Krakauer(4), who noted symptomatic improvement in 16 of 22 hypertensive patients given 688A and what were considered to be significant decreases in supine arterial pressures of the group although dosage and general plan of study were similar. We were especially disappointed in the patient whose pretreatment responses to TEAC suggested that sympathetic nervous mechanisms actively participated in his arterial hypertension. Subsequent persistence of hypertension in the supine position at a time when adrenergic mechanisms had been blocked suggests that at this time, at least, the mechanisms which sustained arterial pressure were not those considered as characteristic of the activity of sympathetic nerves.

Wunch, Warnke and Myers(2) have shown that remissions of the course of malignant hypertension can sometimes be secured by intravenous infusions of Dibenamine; the similar properties of 688A suggest that it should be as effective. However, as noted above, no remarkable effect occurred in 6 patients who received the drug in the terminal phase of malignant hypertension. Thus, in our hands, 688A has not proven of value in the prolonged treatment of hypertensive vascular disease. The limited scope of the study does not exclude the possibility that favorable responses may not sometimes occur nor do our observations bear on the effectiveness of 688A in the treatment of peripheral vascular diseases, or in causalgia(8). Our data suggest that such treatment might advantageously be discontinuous if the effects are to be sustained.

Summary. Oral administration of 688A, an agent which causes adrenergic and sympathetic blockade, did not ameliorate the condition of 8 patients with essential hypertension when administered over a period of from 2 to 26 weeks. A statistically significant, but clinically unimpressive decrease of supine systolic and diastolic pressures occurred in only 1 of these patients; in another, supine arterial pressures were maintained in the presence of "blockade" and tolerance to a daily dosage of 1200 mg was established at the end of 4 weeks.

We thank Mr. William Swain, formerly of Smith, Kline and French, Inc., for generous supplies of the drug and Miss Enid Davy, R.N. for her conscientious assistance in the study.

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Some Observations on Effect of Gamma Radiation on the Biochemistry of the Rat Thymus.* (19592)

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(Introduced by J. M. Coon.)

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The marked sensitivity of thymus to both internal and external radiation has been well established. Morphologic changes(1) can be detected within a few hours after exposure. On the other hand, Barron reported that 4 hours after exposure to x-ray (500 r) the respiratory and glycolytic activities of rat thymus were normal(2); marked changes in respiration and glycolysis were not evident until at least 24 hours after exposure.

It was thus of interest to establish whether various respiratory enzymes of thymus homogenates maintained their activities in spite of extensive cellular damage as evidenced by changes in nucleic acid concentrations.

Methods. In most experiments, 2- to 3-month-old Sprague-Dawley female rats, weighing between 150 and 200 g, were used. It was frequently necessary to pool the thymuses from 3 or more rats in order to obtain sufficient amounts of tissue. Rats were given a dose of 800 r of gamma radiation from a 4 curie point source of Co^{60}. This dose is slightly above the LD_{50}. Succinic dehydrogenase, malic dehydrogenase, cytochrome oxidase, and adenosine triphosphatase were assayed by the methods of Potter and coworkers(3-5). The capacity of thymus homogenatees to esterify inorganic phosphate was studied using a system based on that of Potter(6), except that succinate and pyruvate, both at a final concentration of 0.004 M, were used together as substrates. Neither substrate by itself was adequate for esterification. Attempts were made to prepare mitochondria from thymus by differential centrifugation of tissue homogenized in 0.25 M sucrose(7). The principal difficulty seemed to be in obtaining sufficiently complete separation of nuclei from cytoplasmic elements; up to 40% of the succinic dehydrogenase activity of the thymus

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TABLE I. Effect of Irradiation (800 R) on the Concentration of Certain Enzymes and Nucleic Acids in Rat Thymus.

	Avg values with avg deviations at varying times after irradiation			
	3 hr	6-12 hr	24-48 hr	60-96 hr
		μl/hr/mg dry wt		
Succinic dehydrogenase				
C*	31.5 ± 2.3	25.4 ± 4.8	26 ± 3.5	29.1 ± 6.5
E	29.8 ± 3.1	22.8 ± 4.1	20.2 ± 1.7	20.5 ± 2.4
R	.94	.90	.78	.70
Malic dehydrogenase				
C	11.4 ± 1.4	10.4 ± 2	7.4[1]	—
E	11.7 ± 1.6	10.6 ± 3.6	7.4	
R	1.03	1.02	1	
Cytochrome oxidase				
C	213 ± 29	196 ± 35	240 ± 20	270†
E	219 ± 20	206 ± 28	232 ± 33	260
R	1.03	1.05	.97	.96
		μg P/15 min/mg dry wt		
Adenosine triphosphatase (.004 M Ca^{++})				
C	14.3 ± 1.2	15 ± 1.4	11.7 ± 3	14.3 ± 1.7
E	13.5 ± 1.7	14.7 ± 2.4	8.4 ± .6	12.3 ± 2.2
R	.95	.98	.72	.86
Adenosine triphosphatase (No Ca^{++})				
C	10.8 ± 1.4	12.6 ± 1.6	10.1[1]	12.1 ± .5
E	10.7 ± 1.9	11.9 ± 3.6	6.1	9.8 ± .8
R	.99	.95	.61	.81
		mg P/100 g dry wt		
DNA phosphorus				
C	900 ± 100	952 ± 110	903 ± 160	902 ± 118
E	767 ± 5	695 ± 85	320 ± 180	307 ± 140
R	.85	.73	.35	.34
RNA phosphorus				
C	287 ± 41	353 ± 38	330 ± 43	314 ± 16
E	263 ± 31	292 ± 40	208 ± 16	231 ± 19
R	.92	.83	.63	.74

* C = Control, E = Exposed, R = Ratio E/C.

† One determination.

was sedimented with the nuclei. Most of the remaining activity, not sedimentable at 600 g, was precipitated at 13,000 g, an average of 16% remaining in the supernatant. In 17 experiments, the average succinic dehydrogenase activity of rat thymus mitochondria was 820 μl O_2/hr/mg N. Fractionation of phosphorus in the homogenized thymus was carried out using a combination of the methods of Schmidt and Thannhauser(8) and of Schneider(9).

For experiments with P^{32}, a dose of 100 μc/kg was administered intraperitoneally immediately after irradiation, and the animals sacrificed 3 hours later. Acid-soluble and phospholipid phosphorus were separated by the Schneider procedure as modified by Friedkin and Lehninger(10). Nucleic acids were separated by the Schmidt and Thannhauser procedure(9). DNA was reprecipitated 5 times to achieve constant specific activity. RNA was freed of inorganic phosphorus of phosphoprotein origin by 2 consecutive additions of carrier inorganic phosphate and subsequent precipitations as magnesium ammonium phosphate. Inorganic phosphate of the acid-soluble fraction was similarly measured after 3 precipitations with magnesia. All samples were counted sufficiently long to give a standard deviation of less than 2%, except in the phosphoprotein phosphorus assays.

Results. Table I shows the effect of 800 r

TABLE II. Phosphate Esterification in Normal and Irradiated Rat Thymus.

	No. rats	μg P esterified/20 min/50 mg: Succinate + pyruvate (1)	μg P esterified/20 min/50 mg: No substrate (2)	μg P esterified/20 min/50 mg: Total capacity (1)–(2)	μl O_2/10 min/50 mg: Succinate + pyruvate	μl O_2/10 min/50 mg: No substrate
Controls:						
Avg	10	+15	–21	+36	19.2	3.4
Stand. error		2.52	2.25	3.24	1.08	.89
Exposed:						
Avg	7	–7	–28	+21	18.1	3
Stand. error		5.71	3.10	3.73	.92	.63
"t" values		3.52	1.84		.8	.3

TABLE III. Effect of Irradiation on Incorporation of P^{32} into Compounds of Rat Thymus.

P fraction	Specific activity of P fraction × 10^2 / Specific activity of inorganic P: Control	Specific activity of P fraction × 10^2 / Specific activity of inorganic P: Exposed	Ratio, Exposed / Control
Acid-soluble organic	3.26	2.64	.81
Phospholipid	4.37	4.85	1.11
DNA	6.95	3.26	.47
RNA	7.80	4.72	.60
Phosphoprotein*	13	14	1

* Stand. dev. of counts = 10% of total.

whole-body irradiation on the activity of various enzymes in rat thymus, and on the concentration of total nitrogen, DNA phosphorus, and RNA phosphorus. It is evident that although there was some diminution in activity of both succinic dehydrogenase and adenosine triphosphatase activities, the most dramatic change occurred in concentration of DNA-P. The data at 3 hours after irradiation are averages of 4 experiments; the other time intervals represent 3 experiments. Three control rats and 3 to 6 exposed rats were used in each experiment.

Table II shows the effects of radiation on phosphate esterification. Under the conditions of these experiments, there was no effect on the oxygen consumption 3 hours after irradiation. There was, however, a marked impairment of the capacity of the thymus homogenate to esterify inorganic phosphate from the medium. Some of this effect might be explained on the basis of increased phosphatase activity observed following x-irradiation(11), but the data in column 2 of Table II indicates that the release of inorganic phosphate in the absence of an oxidizable substrate is not increased sufficiently as a result of radiation to account for the major part of the difference between exposed and control thymus; the differences between esterification observed in the presence of succinate and pyruvate, and the increase of organic phosphate seen in the absence of substrates, which may be taken as an indication of the total capacity of the thymus to esterify inorganic phosphate against a dephosphorylation gradient (column 3 of Table II), are also significantly altered as a result of exposure to radiation.

It was thought that the lowered concentrations of nucleic acids, particularly DNA, might be attributable to the impairment in the esterification mechanism indicated above. Therefore, the incorporation of P^{32} into various phosphorus fractions was studied 3 hours after exposure. The effects observed (Table III) are similar to those reported by Lavik *et al.*(12) for the effects of toxic doses of P^{32}, DNA phosphorus showing the most marked depression of incorporation, RNA-P showing a lesser change, and phospholipid phosphorus turnover remaining unchanged. The concentration of phosphorus in the DNA and RNA fractions were altered by radiation to the same extent as in the experiments reported in Table I. The concentration of phospholipid, like the incorporation of P^{32}, was not materially changed.

The specific activities in Table III (counts per minute per mg P) are given in terms of the specific activities of inorganic phosphorus. The total uptake of P^{32} by the thymuses of the exposed rats was only 55% of the control. It was not established whether this difference was caused by decreased penetration of P^{32} into the thymus or by decreased absorption from the peritoneal cavity.

A number of experiments in which mitochondria were isolated from thymuses of exposed animals were attempted, but only 3 were successful in terms of approximating 100% recovery of the succinic dehydrogenase activity of the entire homogenate in the subcellular fractions. In the 3 experiments which could be considered adequate from this standpoint, the succinic dehydrogenase activity of the mitochondria was approximately 20% lower than the activity of mitochondria simultaneously prepared from normal rat thymus.

Discussion. The work of Barron(2,13,14) has indicated the possibility of attributing the deleterious effects of ionizing radiations on animal tissues to an inactivation of sulfhydryl enzymes. The experiments presented here, however, show that none of 3 sulfhydryl enzymes(15) tested was appreciably altered by radiation, when studied as components of a thymus homogenate at a time after exposure when marked morphologic changes had occurred.

The decrease in both turnover and concentration of DNA phosphorus seems to imply nuclear damage which is apparently independent of inactivation of cytoplasmic enzymes (although not necessarily independent of some other effect on the cytoplasm). Barron has reported(2) that 4 hours after exposure of a rat to x-radiation, the glycolytic and respiratory activities of the thymus do not deviate significantly from the control value, although histologic change is well established(1). Beginning 24 hours after exposure, and persisting for 7 or 8 days thereafter, however, the respiratory activity of thymus slices is depressed to 30 to 50% of the normal(3), although we were unable to detect any such order of diminution of activity of the respiratory enzymes which we had studied, using a water homogenate.

We had observed, however, some diminution of succinic dehydrogenase activity of the mitochondria from thymuses of irradiated rats, although these experiments were not conclusive. This observation tends to support the observations of Barron with tissue slices, rather than our other studies with aqueous homogenates in which the integrity of the mitochondria was lost.

In view of recent work by Potter *et al.* (16), it is possible that the discrepancy between the susceptibilities of slices and homogenates to the actions of ionizing radiations may be related to differences in cellular organization, particularly in respect to the mechanism for regulation of levels of high-energy phosphate; ". . . the breakdown of high energy phosphate may be rate-limiting for oxygen uptake in the mitochondria and in the slice, but not in the whole homogenate" (16).

Our results on lack of enzyme inactivation are far from extensive, and certainly do not rule out the possibility that sulfhydryl enzymes other than those studied here may be more sensitive to *in vivo* inactivation. Nevertheless it seems important to emphasize that destruction of sulfhydryl enzymes following the irradiation of biologic material is not a uniform, indiscriminate process; such slight inactivation as was observed in our experiments might lead one to suspect that *in vivo* inactivation of respiratory enzymes through the action of ionizing radiations, either directly or indirectly through activation of water, was not even a particularly important aspect of radiation damage, at least insofar as a direct mechanism of cellular destruction was concerned.

Interference with DNA synthesis would seem to be a more plausible explanation of the immediate cause of cellular damage, the later changes in respiration and glycolysis being merely an effect of diminished or altered cellularity. A decreased capacity of the tissue to esterify inorganic phosphate could account for the decreased concentration and turnover of DNA phosphorus, but one would not expect this impaired esterification to be specific for DNA. A certain specificity does exist, however, in view of the more striking decreases in P^{32} incorporation into DNA than into RNA, with no change in phospholipid turnover, a finding which would not be anticipated if a diminished capacity for phosphorus esterification were the sole metabolic defect produced by radiation. Hence the action through which radiation alters the metabolism of DNA remains obscure.

It is conceivable that the important effects of radiation are directed principally toward cell surfaces, resulting in altered permeability characteristics leading to loss of diffusible cofactors. Such a concept, although capable of reconciling most of the data, is not readily amenable to experimentation in as complex an organism as the rat.

Summary. Negligible changes were observed in the activities of succinic dehydrogenase, malic dehydrogenase, cytochrome oxidase, and adenosine triphosphatase in a thymus homogenate prepared from a rat exposed to 800 r of gamma radiation; however, pronounced diminution both in concentration and in P^{32} incorporation of thymus DNA, and to a lesser extent RNA, were evident 3 hours after exposure of the rat, and became progressively greater during a 96-hour post-irradiation interval. The capacity of a thymus homogenate to esterify inorganic phosphate was appreciably diminished in the irradiated rat. It is suggested that the diminished respiration observed in slices of thymus from irradiated rats is not a primary radiation effect, since comparable changes do not occur in the activity of individual respiratory enzymes studied here.

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Cultivation of *Endamoeba Histolytica* in Embryonic Fluids.* (19593)

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(Introduced by E. C. Faust.)
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Since the first cultivation of *Endamoeba histolytica* by Boeck and Drbohlav(1), the goal of parasitologists has been to maintain this protozoön free of association with other organisms. Thus far all attempts have failed, although within the last few years progress has been reported. Shaffer and co-workers(2-5) cultivated *E. histolytica* in a modified thiogly-

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TABLE I. Cultivation of *E. histolytica* in Embryonic Fluids from Different Sources.

	Amebic population 48 hr after inoculation	
Source of fluids	With traces of blood	Without blood
Human-amniotic, full term	+	—
Dog-amniotic, " "	—	—
Chick embryo-amniotic, >14 days	—	—
Dog-amniotic, 3 to 4 wk	++++	*
Cow-amniotic, 3½ mo	++++	++++
" " 5 mo	+++	++++
Chick embryo-amniotic, 9 to 13 days	++++	++++
Chick embryo-allantoic, 9 to 13 days	++++	*
Chick embryo-amniotic and allantoic mixed, 9 to 13 days	++++	*

—=no amebae seen; +=survival, no apparent multiplication; ++=survival, minimal multiplication; +++=Some multiplication; ++++=definite multiplication.

* Not tested.

collate medium in which bacterial growth was inhibited by the use of penicillin in the medium, while Phillips(6) was able to maintain cultures of *E. histolytica* in which *Trypanosoma cruzi* was the sole associated microorganism. The experiments herein reported were carried out to develop a transparent medium which would 1) provide dependable multiplication of amebae; 2) not require bacteria or other microbial associates, or if required, only in small numbers; 3) permit storage of the medium, as contrasted with the medium of Shaffer *et al.* which must be prepared just before use, and 4) provide large numbers of amebae sufficiently free of bacteria and particulate matter so that precisely controlled studies on the metabolism of these protozoa could be carried out. This medium was to be developed to be used as a research tool and not for laboratory diagnosis.

Materials and methods. Strains of amebae employed. 1) Clone of strain 22, isolated in 1947 from a human brain abscess and used experimentally since 1949 in our laboratory (7). The associated bacterial flora consisted of a species of *Corynebacterium* closely resembling *C. hoffmani* and a second diphtheroid which was considered to be a variant of the above or a member of the same genus obligately parasitic on the first species, since it could not be cultured separately either aërobically or in an atmosphere of phosphorus pentoxide(8). 2) *Strain NRS* grown with a mixed, undetermined bacterial flora. 3) *Strain U.C.* grown with a mixed, undetermined bacterial flora, obtained through the courtesy of Dr. William Balamuth, Northwestern University, Evanston, Ill. 4) *Strain 19t,* obtained by us from a human infection, freed of its bacterial associates and successfully cultured with organism *t*(9). 5) *Strain 103* grown with organism *t,* obtained through the courtesy of Dr. Charles W. Rees, National Institutes of Health, Bethesda, Md. In addition, amebae from other sources were occasionally employed. With the exception of strain 103 *t,* which was cultured routinely in a diphasic whole-egg medium, all strains were maintained in Balamuth's aqueous egg yolk infusion medium(10).

Preparation of media. Embryonic fluids were obtained from chicks, human beings, cows and dogs. For collection of chick fluids 9- to 13-day embryonating eggs were candled and drilled according to the method described by Beveridge and Burnet(11). After the shell was removed and the shell membrane teased away, amniotic and allantoic fluids were removed aseptically with a Pasteur pipet. Amniotic fluids were collected from human sources at cesarean section and by aspiration just before delivery, from one dog after 3 to 4 weeks of gestation and from another dog just before delivery, and from one cow after 3½ months and another cow after 5 months of gestation. Some of these fluids unavoidably contained traces of blood. After collection they were distributed aseptically in 3.8 ml amounts in 125 x 14 mm sterile test tubes.

Results. Cultivation in embryonic fluids from different sources. The amebae used in the preliminary study were from a clone of strain 22. For inoculation from aqueous egg-yolk infusion medium, 0.2 ml of 48-hour cultures was introduced into the various embryonic fluid media. Penicillin and streptomycin were added to make a final dilution of 1,000 units of each per ml of medium. After inoculation the tubes were sealed with petrolatum and incubated at 37°C for 48 hours.

The results (Table I) suggest that the species of animal from which the fluid was taken is not as important for survival and multiplication of the amebae as is the time of embryonic or fetal development at which fluids were collected.

Sterility tests for bacteria were made in each case at 48 hours as follows: An inoculum of 0.05 ml from each of the media was introduced into each of 3 tubes containing respectively 10 ml of brain-heart infusion, 10 ml of thioglycollate broth, and 10 ml of aqueous egg-yolk infusion medium(10), to which pennase had been added, and incubated for 10 days. In all of these media the bacteria of clone 22 have been shown to grow abundantly. Since amounts of the antibiotics sufficient to inhibit the growth of bacteria might be carried along with the inoculum into the subcultures, thus possibly giving a false impression of sterility, the inoculum was occasionally freed of the antibiotics in the following manner: The 48-hour-old amebic cultures were centrifugalized at 1500 r.p.m. for 10 minutes in a conical test tube; 0.05 ml of the sediment was then inoculated into another conical test tube containing 10 ml of sterile saline solution and centrifugalized again at 1500 r.p.m. for 10 minutes; 0.05 ml of this sediment was then inoculated into the 3 bacteriologic media and incubated at 37°C for 10 days. As a further safeguard, 0.05 ml of bacterial suspension from 48-hour clone 22 cultures on aqueous egg-yolk infusion medium was inoculated into each of the 3 bacteriologic media mentioned above, to which penicillin and streptomycin, in amounts equal to a final dilution respectively of 5, 10 and 50 units per ml, were added together with pennase. These doses are the equivalent of at least 2 to 10 times the amount carried over from the amebic cultures which were being tested for sterility. Bacteria always developed in the controls. Yet all amebic cultures in these initial experiments except those in cow's amniotic fluid obtained 5 months after beginning of gestation were negative for viable bacteria after 48 hours of incubation.

Maintenance of cultures. The ease of obtaining chick embryonic fluid plus the promising preliminary results suggested further investigation of these fluids. Attempts were made to maintain cultures of amebae of clone 22 by serial passages in amniotic-allantoic fluid mixtures obtained from 9-13-day-old chick embryos, but in repeated trials the amebae gradually decreased in numbers until after 2 or 3 transfers all cultures became negative for amebae. However, when bacteria from clone 22, either from aqueous egg-yolk infusion cultures or from bacterial cultures on nutrient agar slants, were added with antibiotics to these embryonic-fluid amebic cultures, the amebae could be maintained. Population counts using an inoculum of 2,000 to 3,000 amebae per tube indicated a 5- to 10-fold increase in numbers of amebae at the termination of a 48-hour culture period. In nearly 100 cultures tests for bacterial sterility after 48 hours were negative except for controls to which no antibiotics had been added. (Since the completion of these studies this medium has been experimentally employed in our laboratory by Major Lyman P. Frick, MSC., who has demonstrated a living bacterial population of approximately 200 organisms per ml in 48-hour cultures.) Seitz-filtrates of bacterial cultures failed to support amebic growth. Also an anaerobic environment induced by placing the cultures in an anaerobe jar failed to promote continued amebic growth in serial subcultures unless living bacteria were added at the time of each subculture. The technic for routine maintenance of cultures was as follows: 1. Amniotic and allantoic fluids from chick embryos were pooled and tubed in 3.8 ml quantities. 2. At the time of subculture 0.3 ml of a 48-hour bacterial culture, 0.1 ml of antibiotic mixture, and 0.3 ml inoculum from a 48-hour-old amebic culture were added making a total volume of 4.5 ml. 3. The tubes thus inoculated were sealed by pouring a one cm layer of sterile melted petrolatum directly onto the surface of the culture. 4. The cultures were incubated at 37°C for 48 hours, after which time they were subcultured again in like manner. Bacterial cultures of clone 22 flora were maintained routinely in aqueous egg-yolk infusion medium. The antibiotic mixture consisted of penicillin G and streptomycin sulfate to give a final concentration of 1,000 units of each per ml of culture. After harvesting from the chick embryo, the medium has been stored for one month at 4°C without diminution of its growth-promoting properties for amebae. Further studies revealed that blood in the small amount of approximately 0.01 ml of packed cells, which usually contaminated the fluids during harvest, promoted better protozoan growth than clear fluid. This amount was thereafter routinely incorporated in the medium at the time of harvest.

Cultivation of amebae from different sources. Approximately 0.3 ml of menstruum from a dysenteric dog passing numerous trophozoites in his stools and 0.3 ml from a child's stool containing scanty trophozoites were inoculated into the medium. Also 0.3 ml amounts each of amebic cultures of 5 strains with different monobacterial or multibacterial associates were inoculated into 10 culture tubes of chick embryonic fluids. While 5 of these tubes were left with their original floras only, the other 5 received 0.3 ml each of 48-hour-old culture of clone 22 bacteria, grown on aqueous egg-yolk infusion medium, before being sealed and incubated. The results 48 hours after inoculation indicate (Table II) that all of the strains of amebae to which the flora of clone 22 had been added grew in this medium, while only 2 of the strains tested grew solely with their own bacterial associates. Additional studies revealed that strains NRS and 103 could be maintained in this medium provided that they were supplemented at each subculture with 0.3 ml of clone 22 bacteria as indicated above.

TABLE II. Cultivation of *E. histolytica* from Different Sources in Chick Amniotic-Allantoic Fluids.

Sources of amebae	Amebic population 48 hr after inoculation With original flora	 With addition of Clone 22 flora
Clone 22	+++	+++
Dog's dysenteric menstrum	+++	*
Child's feces	++	*
Strain NRS	—	+
" 103 t	—	+++
" U.C.	+++	+++
" 19 t	—	+++

— = no amebae seen; + = survival, minimal multiplication; ++ = some multiplication; +++ = definite multiplication.

* Not tested.

Discussion and summary. 1. These experiments indicate that *Endamoeba histolytica* trophozoites can be cultured successfully in embryonic fluids. Amniotic and allantoic fluids from 9- to 13-day-old chick embryos have given the most consistent results. While growth of amebae occurred in aerobic as well as anaerobic environments, more dependable results were obtained under the latter condition. The fluids retained their ameba growth-supporting qualities after storage up to 30 days, but not after Seitz filtration. 2. The extremely low bacterial population has made large numbers of amebae available for physiologic studies. It is generally realized that bacterial interference must be minimized before metabolic studies of a fundamental nature can be undertaken.

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Effect of Heparin and Treburon in Postprandial Hyperlipemia. (19594)

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Since Hahn(1) demonstrated that heparin cleared alimentary hyperlipomicronemia, much work has been done to elucidate the mechanism for this action(2-4). Although it is as yet uncertain whether lipomicronemia is cleared by conglutination, dispersion, or a combined effect induced by a heparin-plasma protein complex, nevertheless the reaction is consistent and serves as a measure of heparin action upon serum lipids. The present study was concerned with the comparative effects of heparin* and Treburon† (a new synthetic heparinoid) on the physical state of serum lipids using as an index their action on alimentary lipemia. The effect on cholesterol and lipid phosphorus was also determined.

Method. The study included a total of 24 subjects. Ten patients, 2 hospitalized for rheumatic fever, 2 for myocardial infarction, 3 for pneumonia, one for bronchogenic carcinoma, one for cirrhosis, one for obesity, were given 25 g fat meals on 3 successive days. Three hours after the meal (while the lipomicronemia was reaching its maximum phase) 500 mg Treburon was given intramuscularly or sublingually, or 100 mg heparin was given intramuscularly. Medications were changed daily so that 9 of the 10 patients received all 3 medication procedures, but not in the same sequence (one patient left the hospital before heparin administration). Seven patients, 6 of whom received a 25 g fat meal, had cholesterol, cholesterol ester and lipid phosphorus determinations performed, as well as lipomicron counts before and after Treburon administration. The seventh patient of this group was fasting when these studies were undertaken. Treburon was given during the lipemic phase of digestion (6 received 500 mg intramuscularly and one 1000 mg sublingually). Three patients received similar studies using 100 mg heparin intramuscularly. Four patients served to demonstrate the variation in the normal curve of lipomicronemia after a 25 g fat meal. Each of these patients was subsequently given 250 mg Treburon intravenously 2 hours after the test meal and the effect on lipomicronemia noted prior to medication and 5, 15, and 60 minutes after medication. The lipomicronemia was determined on samples obtained from capillary blood drawn into vaccine tubing. Leaving one-third of the tube empty to prevent heat coagulation the clear end of the tube was flamed shut and the miniature test tube thus produced was centrifuged at 2,000 r.p.m. to separate serum from clot. The entire serum sample was transferred to a detergent-free, scrupulously clean glass slide by breaking the tube at the serum clot junction. A cover slip was affixed and with a drop of immersion oil on the cover slip as well as the darkfield substage examination was made using a 95 X objective and a 20 X ocular. One large square of a net micrometer inserted into the ocular was used to make the count in a single focal plane at 100 sites. To avoid subjective effects personnel doing the counts knew neither patient nor medication used. The technic was essentially that previously reported(5) except that due to the intense lipomicronemia, chylomicron index determinations (per cent of visible particles larger than 0.5 μ) could not be accurately determined. The cholesterol and cholesterol ester determinations were done according to a modification of the technic of Sperry and Schoenheimer(6). The lipid phosphorus determinations were performed according to the modified method of Youngburg and Youngburg(7).

Results. Moderate to marked reduction in lipomicronemia was produced in all 24 patients given intramuscular or intravenous heparin or Treburon (Tables I and II, and

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* Heparin was furnished in generous quantities through courtesy of Upjohn Co., Kalamazoo, Mich.

† Treburon is the trademark of the sodium salt of sulfated polygalacturonic acid methyl ester methyl glycoside developed in the laboratories of Hoffmann-La Roche, Inc., Nutley, N. J., furnished by Dr. Leo A. Pirk.

TABLE I. Effect of Heparin and Treburon on Chylomicronemia. Total visible fat globules per 100 squares.

		Intramusc. Treburon (500 mg)			Intramusc. Heparin (100 mg)			Sublingual Treburon (500 mg)		
Name	Age	3 hr p.c.	3½ hr p.c.	4 hr p.c.	3 hr p.c.	3½ hr p.c.	4 hr p.c.	3 hr p.c.	3½ hr p.c.	4 hr p.c.
C.R.	59	520	340	200	730	360	380	570	630	710
S.M.	75	580	110	160	650	185	205	400	680	520
J.M.*	33	760	470	600	670	230	550	690	850	610
C.S.	55	188	212	50	—	—	—	410	310	220
E.S.	53	780	760	400	1500	800	750	1080	365	305
H.H.	63	840	180	605	268	116	132	1050	930	920
M.T.	54	850	124	176	—	235	300	1110	1270	820
J.G.	67	420	180	244	525	330	175	780	760	400
C.L.	78	800	410	64	850	80	80	710	500	520
E.D.	28	1160	145	110	950	180	124	790	810	880

Treburon and heparin were given 3 hr p.c. 25 g fat med.

* This patient weighed 425 lb.

p.c. stands for post cibum (after eating).

TABLE II. Effect of Heparin and Treburon on Chemical and Physical State of Serum Lipids.

		Cholesterol		Lipid	Fat	Cholesterol		Lipid	Fat
Name	Age	Total, mg %	Free, mg %	phosphorus, mg %	particle, 100 sq	Total, mg %	Free, mg %	phosphorus, mg %	particle, 100 sq
		Before Treburon (500 mg IM)				1 hr after Treburon			
F.S.	58	220	86	—	352	205	95	—	164
R.H.	58	301	217	13.8	1180	324	186	14.2	156
J.H.	74	117	83	8.1	920	119	65	7.6	110
C.H.	67	143	55	—	990	143	55	—	188
W.M.	46	155	84	14.6	1060	142	48	13.8	290
A.S.*	45	253	175	11.3	195	256	185	11.7	196
E.D.†	28	172	70	—	450	146	67	—	930
		Before heparin (100 mg IM)				1 hr after heparin			
F.H.‡	68	167	—	7.2	69	167	—	7.2	47
J.H.	74	114	75	7.8	360	107	75	7.9	25
W.M.	46	229	55	23.2	875	217	46	22	305

Heparin and Treburon were given 3 hr p.c. 25 g fat med.

* Subsequently lipemia occurred between 3rd and 4th, not 2nd and 3rd hr.

† 1000 mg Treburon given sublingually, not intramusc.

‡ This subject was fasting.

Fig. 1). Sublingual Treburon in the doses used was ineffective in 7 of 10 patients, and significantly effective in only one. This was similar to the experience with sublingual heparin and Treburon on clotting time determinations(8). Table II demonstrates that the effect of heparin or Treburon on the physical state of postprandial serum fat emulsions is not related to any consistent change in cholesterol, cholesterol ester, or lipid phosphorus content.

Intravenous Treburon produced a clearing of postprandial lipomicronemia within 5 to 15 minutes, and this effect reached a maximum within one hour (see Fig. 1). The normal lipomicron pattern of these patients following a fat meal demonstrates that the counts were static (one) or increasing (three) between the second and third hour. These findings are in accord with those reported in previous studies(5,9).

Discussion. Moreton(10) and Necheles *et al.*(11) have felt that the postprandial lipomicronemia is more prolonged and the chylomicronemia of a greater degree in myocardial infarction patients than in normal subjects. Zinn and Griffith(5) were unable to confirm this by the technic they employed. However, if such a relationship does exist the observed effect of heparin should be beneficial. While denying any essential role in atherosclerosis to chylomicrons, Gofman and

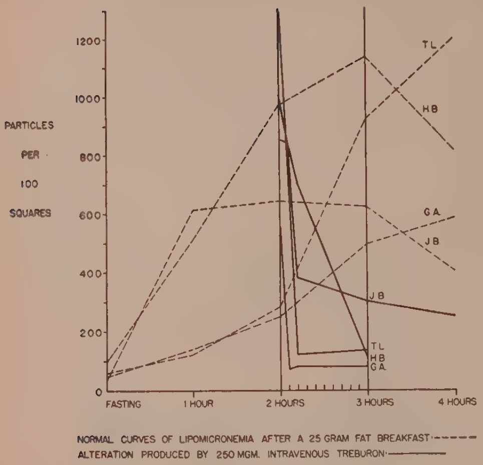

FIG. 1.

associates(12,13) have demonstrated that their atherogenic cholesterol-bearing lipoprotein molecules of the S_f 12-20 class are suppressed by heparin administration. In addition, they have presented evidence that angina pectoris was relieved for prolonged periods by administering heparin twice a week. The relationship between the chylomicron index and S_f 10-20 alterations is now under investigation in these laboratories. The significant reduction in postprandial lipomicronemia by heparin may serve as an indicator of the active principle which alters the cholesterol bearing lipoproteins Gofman has related to atherosclerosis. The findings recorded herein indicate that Treburon affects the observed physical state of serum lipids in a manner identical with heparin, and suggests that it may be substituted for heparin in clinical states where heparin has been found effective.

Conclusions. 1. Heparin or Treburon given intramuscularly were equally effective in producing a sharp fall in alimentary lipomicronemia within 30 to 60 minutes. 2. The alterations noted in serum fat emulsions were not related to changes in cholesterol, its fractions, or lipid phosphorus. These chemical determinations were not significantly influenced by either heparin or Treburon. 3. Treburon given intravenously produced a sharp drop in the lipid particle count within 5 to 15 minutes and this was maximal within one hour of injection. 4. Treburon given sublingually was ineffective.

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Effect of Multiple Inoculations on Interference Between St. Louis Encephalitis Virus and Equine Encephalomyelitis Virus. (19595)

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In a previous communication from this laboratory it was reported that young rats, which are more or less uniformly susceptible to intranasally inoculated Western equine encephalomyelitis (W.E.E.) virus, were much less susceptible to nasally instilled W.E.E. virus if a single intranasal inoculation of St. Louis encephalitis (S.L.E.) virus had preceded by 72 hours the inoculation of W.E.E. virus(1). It was found that 50% of 3- to 4-week-old rats which had had S.L.E. virus instilled into their noses survived a subsequent intranasal inoculation of W.E.E. virus. Since a single intranasal inoculation of S.L.E. virus protected 50% of the animals against W.E.E. virus, further experiments were conducted to determine whether a greater degree of protection would be afforded by multiple intranasal inoculations of S.L.E. virus and also to determine whether heat-inactivated S.L.E. virus would afford protection. The purpose of this communication is to report the results obtained in these studies.

Materials and methods. Viruses and animals. The strain of S.L.E. virus, as well as the strain of W.E.E. virus, used in the present study has already been described(1). The virus suspensions used were prepared from virus-infected brains of 3- to 4-week-old Swiss mice. The infected brains were ground in a mortar with sand and sufficient inactivated, undiluted rabbit serum to make a 10% suspension of the infected brains. The suspensions were centrifuged at about 2000 r.p.m. for 5 minutes to sediment gross particles. Immediately after centrifugation the supernatant fluid was removed and distributed in 2 ml amounts in screw-capped vials. The virus suspension in the vials was "quick-frozen" by rolling the vial in a mixture of dry-ice and alcohol. The frozen virus was then stored at about —70°C in dry-ice cabinet. When needed it was thawed by placing a vial of frozen virus in a water bath at 37°C until the material was thawed. A single suspension of each of the 2 viruses was used. The mouse intracerebral LD/50 titer(2) of the suspension of W.E.E. virus, after storage at about —70°C for 4 days, was $1 \times 10^{-7.8}$. The S.L.E. virus, after 5 days storage in the dry-ice cabinet, had a 50% end point of $1 \times 10^{-8.5}$. The 10% suspension of normal mouse brain (N.M.B.) used was prepared and stored as indicated above except that normal mouse brains were used. The heat-inactivated S.L.E. virus was prepared by heating approximately 5 ml of S.L.E. virus at 56°C for one hour. This virus had been stored at about —70°C for 20 days before it was heated. Eight 3- to 4-week-old mice survived for 21 days following intracerebral inoculation of 0.03 ml of this heated virus. The rats used were male Sprague-Dawley. The mice used in the studies were obtained from Rockland Farms.

Experimental. In the present series of experiments 95 rats 3 to 4 weeks of age were used. The various materials (Table I), inoculated intranasally into these animals, were in the form of 10% suspensions. The 95 rats were divided into 5 groups. One group, consisting of 18 rats, received 0.05 ml of the N.M.B. suspension and 72 hours later received 0.05 ml of W.E.E. virus. A second group composed of 20 rats, received 0.05 ml of the suspension of S.L.E., and 72 hours later 0.05 ml of W.E.E. A third group, consisting of 19 rats, received 3 daily inoculations of 0.05 ml of N.M.B. followed 24 hours after the third inoculation with 0.05 ml of W.E.E. A fourth group of 19 animals was inoculated on each of 3 successive days with 0.05 ml of heat-inactivated S.L.E. and 24 hours later received 0.05 ml of W.E.E. The last group, consisting of 19 rats, received 0.05 ml of S.L.E. virus on

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TABLE I. Interference Between S.L.E. and W.E.E.[*] Comparison between single and multiple inoculations of interfering virus.

No. of rats tested	Material instilled into nose	Interval (days) between W.E.E. and death 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	Results	% mortality
18	N.M.B.×1 W.E.E.	0[‡]	0	16	1	0	0	0	0	1										18/18[§]	100
20	S.L.E.×1 ”	0	0	1	0	0	0	0	1	0	0	1	0	1	1	1	0	0	1	7/20	35
19	N.M.B.×3 ”	0	0	17	1	0	0	1												19/19	100
19	S.L.E.[†]×3 ”	0	0	14	1															15/19	79
19	” ”	0	0	0	0	0	0	0	0	0	0	0	1							1/19	5

[*] N.M.B., normal mouse brain; W.E.E., Western equine encephalomyelitis virus; S.L.E., St. Louis encephalitis virus.

[†] St. Louis encephalitis virus heated 56°C 60 min.

[‡] Zero indicates no deaths that day. Other number indicates No. of rats which died that day.

[§] Denominator denotes No. of rats tested, numerator No. of deaths.

each of 3 successive days and then 0.05 ml of W.E.E. 24 hours after the last injection of S.L.E. The experiments were so designed that all of the animals shown in Table I were inoculated on the same day and with the same batch of W.E.E. virus. Reference to Table I shows that the previous intranasal inoculation of N.M.B. did not protect the rats against W.E.E. virus. This was true for the animals which received 3 inoculations of N.M.B. as well as for those which received a single inoculation, since of 37 rats receiving either single or multiple inoculations of N.M.B., all succumbed to a fatal infection caused by W.E.E. virus. These findings were expected and merely confirm results already reported (1). On the other hand it will be seen that animals which had S.L.E. virus instilled into the nose before the instillation of W.E.E. virus were much less susceptible to W.E.E. virus. Of the 20 rats which received a single intranasal inoculation of S.L.E. virus 72 hours before the inoculation of W.E.E. only 7 or 35% of the animals succumbed to infection with the latter virus. These results compare favorably with the results previously published(1), where it was found that a single intranasal inoculation of S.L.E. would protect 50% of rats so treated against W.E.E. inoculated intranasally. While a high degree of protection against W.E.E. was noted when a single inoculation of S.L.E. preceded the W.E.E. a still greater degree of protection was noted when 3 daily inoculations preceded the W.E.E. Only 1 of the 20 animals which received S.L.E. 3 times before the W.E.E. was inoculated, died. It should be pointed out that in this series of experiments, as well as in those already published(1), that when rats which had had S.L.E. virus instilled into the nose prior to nasal instillation of W.E.E. do succumb they survive for a longer period than rats which had not received S.L.E. Reference to Table I shows that rats which had received 3 intranasal inoculations of heat-inactivated S.L.E. were still highly susceptible to W.E.E. virus although 4 of the 19 animals in this group survived. The explanation for these results may be that all of the virus was not completely inactivated during the heating period and that the rats which were protected were protected by active virus. It will be recalled, however, that the heated S.L.E. virus was tested for activity by inoculating the material intracerebrally into each of 8 mice and was found to be inactive.

Discussion. The data reported here confirm and extend the observation that St. Louis encephalitis virus, instilled into the noses of young rats, changes the susceptibility of these animals to Western equine encephalomyelitis virus subsequently inoculated by the nasal route. In a previous communication(1) it was reported that 50% of rats which had had St. Louis encephalitis virus instilled into their noses resisted infection with Western equine encephalomyelitis virus inoculated intranasally 72 hours later. It was postulated that, in those animals which survived, the St. Louis encephalitis virus, which had been dropped into the nose, had penetrated all of the olfactory nerve endings which project into the olfactory mucosa and had "blocked" the entry into these fibers of the subsequently inoculated

Western equine encephalomyelitis virus. On the other hand, however, if the St. Louis encephalitis virus did not penetrate all of the nerve endings, in certain of the rats, the subsequently injected Western equine encephalomyelitis virus could reach the brains of these rats over a limited number of nerve fibers and cause a fatal encephalitis. That the Western equine encephalomyelitis virus did reach the brain by passing over a limited number of nerve fibers was suggested by the observation that in those animals which did succumb, the incubation period was longer and the animals survived for a longer period than did controls which had not received St. Louis encephalitis virus before the Western equine encephalomyelitis virus was injected. In the experiments reported here it was found that a single intranasal inoculation of St. Louis encephalitis virus protected 65% of the rats against Western equine encephalomyelitis virus. However, when rats were given 3 inoculations of St. Louis encephalitis virus before the equine encephalomyelitis virus was inoculated a greater degree of protection was noted since 95% of rats so-treated survived. From the findings reported here, as well as elsewhere(1) it would appear that St. Louis encephalitis virus, having passed over the fibers of the olfactory nerve and being present in the brain, "interferes" with the subsequent passage of Western equine encephalomyelitis virus over these fibers and thus prevents the latter virus from reaching the brain. The degree of interference and thus the percentage of animals that survive can be increased if 3 rather than 1 inoculation of St. Louis encephalitis virus precedes the inoculation of Western equine encephalomyelitis virus.

Summary. A single intranasal inoculation of St. Louis encephalitis virus protected 65% of 3- to 4-week-old rats against Western equine encephalomyelitis virus subsequently inoculated by the same route. The percentage of animals protected was increased to 95% if 3 inoculations of St. Louis encephalitis virus preceded the Western equine encephalomyelitis virus.

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A Biological Method for Determining Small Quantities of Sodium Retaining Substances.* (19596)

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Numerous investigations on the retention of urinary sodium for the quantitative estimation of adrenal cortical extracts and desoxycorticosterone have been made in adrenalectomized dogs(1-5), mice(6), and rats(7,8). Several workers(9,10) have taken advantage of the increased potassium excretion in rats treated with desoxycorticosterone as a means of estimating salt-regulating activity. Widespread investigation of the effect of desoxycorticosterone on sodium and potassium excretion has been somewhat restricted by the lack of a practical test, and a satisfactory experimental animal.

This report is concerned with sodium excretion in the adrenalectomized rat in response to various dose levels of desoxycorticosterone acetate with a constant load of administered sodium.

Material and methods. Biological. Male albino rats, obtained from Holtzman-Rolfsmeyer Co. (Madison, Wis.) and weighing from 150-155 g, were adapted to general laboratory conditions for 48 hours in group

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TABLE I. Sodium Excretion and Urine Volume Changes of Adrenalectomized Rats Given Desoxycorticosterone Acetate.

DCA, μg	No. animals	mg Na excr., avg ± S.E.	Na retained mg	Na retained %	ml urine vol, avg ± S.E.	Urine vol, % change
0	19	2.65 ± .13	—	—	1.21 ± .11	—
1	9	2.58 ± .18	.07	2.5	1.09 ± .13	— 9.9
2	12	1.88 ± .15	.77	29.1	.97 ± .09	—19.8
4	11	1.40 ± .12	1.25	47.2	.87 ± .12	—28.1
6	9	1.09 ± .15	1.56	59	1.34 ± .20	+10.7
12	6	.30 ± .04	2.35	88.7	.75 ± .20	—38

cages, after which time they were further adapted for about 24 hours to individual cages. Following the period of adaptation, the animals were bilaterally adrenalectomized under ether anesthesia. They were fed Rockland Farm rat pellets *ad libitum* both pre- and postoperatively. Tap water given to unoperated animals was replaced by a 5% Cerelose (corn sugar) solution for the adrenalectomized rats. Eighteen and one-half hours after the last adrenalectomy (designated as zero hour), the animals were deprived of food pellets, but were allowed Cerelose solution for an additional 4.5 hour period. Distilled water replaced the Cerelose solution at the 23rd hour, and constituted the sole source of water intake through the remainder of the test. On the 24th hour after the last operation, one hour after removal of the Cerelose solution, each control animal was injected subcutaneously in the shoulder region with 0.05 ml corn oil, followed by a subcutaneous injection of 2.5 ml of a freshly-prepared 0.85% solution of sodium chloride (equivalent to 8.29 mg sodium) divided at 2 sites in the shoulder region. Other animals were similarly injected with 0.05 ml oil solutions containing desoxycorticosterone acetate (DCA) at various dose levels, followed by the salt injection. Three hours later at the 27th hour, a second volume of 0.05 ml corn oil, or oil solution of DCA, was similarly injected into the shoulder region of the respective groups of animals. The total volume of oil, or oil solutions of DCA, injected in 2 stages was 0.1 ml, and the dosage level of the hormone was expressed in terms of total μg of DCA in this volume. One hour following the oil or DCA injections the rats were anesthetized with ether. The urinary bladder was emptied by suprapubic pressure and by the effects of the ether *per se*, but the bladder was not rinsed. While the rat was anesthetized the penis was extruded from its loose prepuce with the aid of forceps and held with a suspended towel clamp. The urethra in the region of the glans penis was ligated twice with No. 10 cotton thread, and after release of the clamp the penis was replaced in its original position. Each animal was returned to its individual cage, without food, but continued with distilled water, for exactly 2 hours. At the end of this period (the 28th to the 30th hours), the animals were sacrificed by cervical dislocation, and the urinary bladder was exposed. The basal portion of the dilated urinary bladder was firmly clamped with a pair of forceps, and the bladder removed by an undercut with a pair of scissors. The bladder, still securely clamped, was rinsed in a beaker of distilled water to remove blood and body fluids, lightly blotted to rid it of excess water, then transferred to a small funnel stoppered with a cotton pellet. The urine was filtered into a 5 or 10 ml burette which contained water at a known level for measurement of small quantities of urine. After measurements of the urine volume by difference, the contents of the burette were emptied into graduated 50 ml centrifuge tubes. The bladder was opened by several cuts with a pair of scissors and the bladder, forceps, scissors, funnel and burette were thoroughly rinsed with small portions of distilled water. The cotton pellet was similarly rinsed several times, alternating flooding and compression. All rinsings were added to the urine sample in the 50 ml centrifuge tube and diluted to the 25 ml mark after protein and phosphate precipitations.

Chemical. The total amount of sodium excreted in each sample was determined colorimetrically(11).

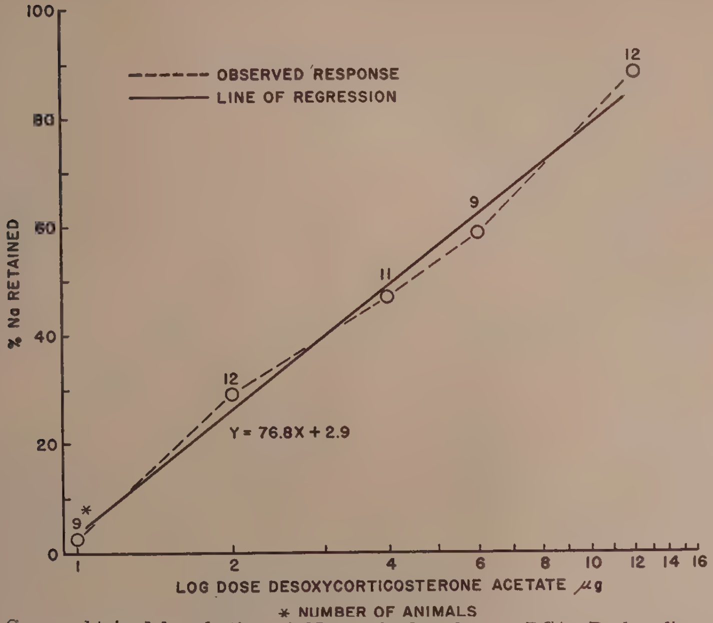

FIG. 1. Curves obtained by plotting % Na retained *vs* log µg DCA. Broken line represents observed response and solid line the line of regression.

Results and discussion. The results of the urine sodium and volume changes in the control and DCA-treated groups of animals are summarized in Table I. An inspection of the table will show that the bioassay of DCA described here, utilizing bilaterally adrenalectomized rats is an extremely sensitive one. Highly significant retention of urinary sodium ($P = < 0.01$) was obtained with total doses of 2 µg or more of DCA, as compared to the oil-injected groups of animals. With 2 µg of DCA, sodium excretion was reduced from an average of 2.65 mg in the control group to 1.88 mg, or to the extent of 0.77 mg. Progressive increases in the levels of DCA caused corresponding increases in retention of sodium. Significant changes ($P = < 0.05$) in sodium excretion were observed between the doses of 2 and 4 µg of DCA, and between 4 and 6 µg of DCA. Highly significant "t" values of 3.66 and 4.96 were obtained for the differences in sodium excretion between 2 and 6 µg DCA, and between 6 and 12 µg DCA, respectively.

Urine volume change of significance occurred between the oil-injected control and 4 µg DCA treated groups of animals ($P = < 0.05$). No significant urine volume changes were observed with other levels of DCA relative to the control group of animals. Differences in the urine volume of animals given the 6 and 12 µg doses of DCA, and between those given 4 and 6 µg DCA, were analyzed statistically, but no significant differences were found.

Plotting the log of the dose of DCA against the percentage sodium retained, a curve with points essentially on a straight line (Fig. 1, broken line) was obtained. The line of regression (Fig. 1, solid line) was determined from the various mean percentages of sodium retained with doses from 1 to 12 µg DCA, by use of the equation $y = y + b(x - \bar{x})$ as discussed by Fisher(12) and by Burns(13), with b representing the slope of the line, y the percentage of sodium retained, $\bar{y}$ the mean value of y, x the log dose of DCA, and $\bar{x}$ the mean value of x. The value of b was calculated from the formula $b = \Sigma y(x - \bar{x})/\Sigma(x - \bar{x})^2$.

Calculation of the index of precision ($\lambda = s/b$, where s is the average standard deviation in response independent of dose and b is the slope of the curve) indicates a satisfactory degree of precision for the method ($\lambda = 0.256$). The biological technic as presented here is a convenient short-term test which requires a lapsed time of about 30 hours to complete, exclusive of the maintenance of the animals for adaptation, adrenalectomy, and analysis for sodium. With such a short-term test, however, one must observe strict adherence to scheduled procedures. The method of assay as described precludes the requirement for special technical skills, and eliminates the need for special apparatus.

Dorfman, Potts, and Feil(7) reported significant retention of radiosodium with 1 μg of desoxycorticosterone in adrenalectomized male rats. More recently, Deming and Luetscher (8) proposed a bioassay for desoxycorticosterone-like substances sensitive in the order of 10 μg of desoxycorticosterone acetate (DCA) utilizing selected adrenalectomized female rats. The authors indicated, however, that variations in the urinary excretions of sodium in different groups of animals make necessary a preliminary testing with DCA to insure accurate evaluation of a test material. Spencer(6), utilizing adrenalectomized mice, selected on the basis of capacity to excrete 70 to 130% of a sodium load within 6 hours, demonstrated 15% sodium retention in cross-over tests with a dose of about 0.5 μg of desoxycorticosterone acetate per 30 g mouse. An inconvenient feature of the biological assays of Spencer(6), and Deming and Luetscher(8) rests on the problem of preliminary selection of animals. The high incidence of adrenal cortical rests in some strains of mice (14) decreases their usefulness as test animals. Simpson and Tait(15) in dose-response studies on the effect of desoxycorticosterone acetate on radiosodium excretion in adrenalectomized rats, observed a decrease in the sodium excretion rate over that of the controls with only 0.5 μg of the hormone. No tests for significance were reported.

Deming and Luetscher(8) reported on the problems of supply, variable activity, measurement of radiosodium, and daily variations in the uncontrolled sodium intake of the animals for the method of Dorfman, Potts, and Feil(7). The problems of technical skill and special apparatus, make the method of Dorfman, Potts, and Feil(7), and of Simpson and Tait(15) less practical from the standpoint of general bioassay procedures than the method presented here. An early study on potassium excretion in rats with the salt-regulating hormone was reported(9). Dorfman(10) observed significant increases in urinary radio-potassium chloride in amounts of 146 to 280 μg (containing radio-potassium) per g of body weight. The indications are, however, that further studies would be required for the development of a quantitative assay of desoxycorticosterone activity.

Summary. 1. A practical micro-bioassay technic for desoxycorticosterone acetate activity is proposed, using albino, urethra-ligated, bilaterally adrenalectomized, male rats of 150-155 g weight. 2. A calibration curve is demonstrated using desoxycorticosterone acetate as the standard hormone and the percentage sodium retained as the measure of its activity. Two μg DCA injected subcutaneously in 2 stages produce a highly significant retention of administered sodium. 3. The proposed bioassay requires 30 lapsed hours and a minimum of special technical skills and apparatus, but a high degree of sensitivity and a satisfactory degree of precision are indicated. 4. No consistent correlation was found between urine volume changes and DCA dosage levels. A significant change in urine volume was observed with 4 μg DCA relative to the control group of animals, but not with the higher or lower doses of DCA studied.

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Production of Pigments Similar to Those from Hair from Amino Acid and Keratin Interaction. (19597)

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Arnow(1) found that a red pigment obtained from red human hair by continuous boiling with 0.1 N hydrochloric acid for 3 days or longer has the same absorption spectrum as solutions of oxidized (red) dopa melanin, and concluded that the red pigment is a natural oxidation product of melanin. However, Lee and Penrose(2) obtained a red pigment from the hair of a human albino by the same method and suggested that the color is not of natural origin, but is produced from some colorless chromogen in the hair.

Although soluble pigment is formed during the hydrolysis of the keratin in whole hair with strong mineral acids(3,4), neither isolated hair melanins nor solutions of hair keratin yield a soluble pigment on treatment with acid. This suggests that the soluble pigment may be the product of reactions involving intermediate keratin hydrolysis products. To determine whether keratin may be associated with pigment production from colorless chromogens in the hair, tyrosine and tryptophan were boiled in mineral acids with and without nail keratin and the resulting colors were compared. Tyrosine and tryptophan were used as chromogens since there is evidence that they may be precursors of mammalian epidermal pigments(5,6), and nail keratin because it is pigment free.

Experimental. Washed nail keratin was defatted with ether and weighed amounts were added to known concentrations of *L*-tyrosine and *L*-tryptophan in solution in 0.1 N and 6 N hydrochloric acid and 6 N sulfuric acid. The solutions were heated either continuously or intermittently under reflux for 15 hours or longer and were then allowed to stand in the light for 60 hours, during which time observations were made. Details of the treatments are summarized in Table I. Spectrophotometric readings of the red tryptophan-keratin solutions were made in a Beckman spectrophotometer. All readings were made at least 84 hours after the beginning of treatment. Chromatographic isolation of the tryptophan-keratin pigment was attempted. Adsorption on talc and Celite columns (1:1 by volume) produced sharp bands which were easily eluted with 0.2 N sodium hydroxide.

Results. The results of acid treatment are summarized in Table I. Keratin affects the color potentialities of both tyrosine and tryptophan when these are boiled with concentrated mineral acids (Exp. 2, 3, 5, and 6) and of tryptophan when boiled with dilute HCl (Exp. 2, 5, and 8). Color appears more rapidly in tryptophan solutions in HCl when keratin is present (Exp. 2, 5, and 8). There appears to be a threshold concentration for the production of color from tryptophan alone in 0.1 N HCl (Exp. 2 and 9). Black pigment formation from tyrosine is seen clearly only when tyrosine is treated with 6 N H_2SO_4 in the presence of keratin (Exp. 6) but slight color differences in 6 N HCl solutions (Exp.

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TABLE I. Differences in Pigment Production When Tyrosine and Tryptophan Are Boiled with Acid 15 Hr with and without Nail Keratin. The colors recorded are of solutions at 72 hr after beginning of treatment. Dash indicates absence of color. Numbers in parentheses indicate time in hr at which color was first observed.

Exp. No.	Material added, mg/ml solvent	Solvent		
		N/10 HCl	6 N HCl	6 N H_2SO_4
1	0	—	—	—
2	.5 mg tryptophan	—	Red-yellow, dark cast (5)	Bright yellow (2)
3	.5 mg tyrosine	—	—	—
4	2.5 mg keratin	—	Faint yellow* (5)	Faint yellow* (5)
5	.5 mg tryptophan and 2.5 mg keratin	Yellow (72)	Dark red-brown (2)	Red-brown; ppt of red-brown particles (2)
6	.5 mg tyrosine and 2.5 mg keratin	—	Faint yellow* with dark cast (5)	Yellow-brown; black ppt (2)
7	.25 mg tryptophan and .25 mg tyrosine	—	Red-yellow, darker cast than exp. 2 (5)	Red-brown, less red than exp. 5; flocculent red-brown ppt (10)
8	.25 mg tryptophan, .25 mg tyrosine and 2.5 mg keratin	Yellow (72)	Dark red-brown (2)	Red-brown with flocculent red-brown ppt; same as exp. 7 (2)
9	1 mg tryptophan	Yellow (72)	Yellow-red (4)	
10	.25 mg tryptophan and 1.25 mg hydrolyzed keratin (sol. 4)		No color change in 5 hr	No color change in 5 hr
11	.25 mg tyrosine and 1.25 mg hydrolyzed keratin (sol. 4)		Same	Same

* Yellow color due to the presence of hydrolyzed keratin in solution.

4 and 6) indicate that some pigment may be formed during this treatment. Completely hydrolyzed keratin does not induce rapid color changes as does unhydrolyzed keratin (Exp. 10 and 11).

Mixed solutions of keratin and tryptophan, both of which have been treated separately with 6 N HCl, show no distinct color changes before 9 days. Then the solutions have a purplish-blue color, which changes to red-brown within 15 minutes on heating. The bluish color resembles the transitory color which appears on the keratin particles during the first few minutes of boiling with tryptophan in 6 N HCl. This similarity, and the rapid color change in hydrolyzed keratin solutions on heating, suggest that similar reactions may occur in both solutions.

The absorption curve of the tryptophan-keratin solutions in 6 N HCl which was obtained using 6 N HCl as the standard is not appreciably different from that of either oxidized melanin or the red pigment obtained from hair by Arnow (Fig. 1). This suggests that the pigment from hair may be formed from some component, perhaps tryptophan, in the hair keratin during the prolonged acid treatment. The similarity of absorption pattern between solutions of the red pigment and of oxidized melanin may be due in part to some similarity of the light scattering particles in the 2 solutions.

Curves of both the 0.1 N and 6 N HCl solutions of tryptophan and keratin were obtained using as standards solutions containing the same concentrations of tryptophan and keratin, both of which had been treated separately. The 0.1 N acid solutions showed an absorption band with a sharp peak in the ultraviolet at 354 mμ, and the 6 N acid solutions showed a band with a maximum at 535 mμ and a minimum at 465 mμ (Fig. 2). Since solutions of keratin and tryptophan treated separately do not, when combined, show the same absorption as solutions having the same concentrations of keratin and tryptophan boiled together, an interaction product must be formed when the two are treated together which contains, in addition to the reddish brown pigment from tryptophan, some factor from keratin.

The interaction product resulting from

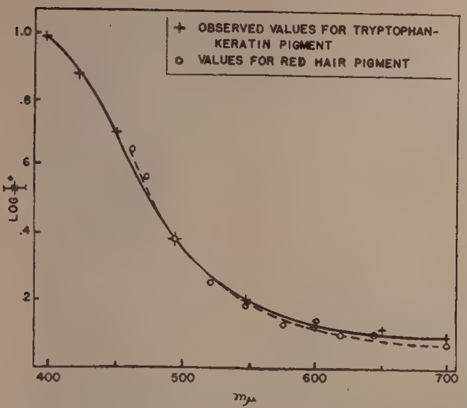

FIG. 1. Absorption spectrum of solutions of tryptophan and keratin treated with 6 N HCl obtained when read against the solvent (solid line) and the spectrum obtained by Arnow(1) for acid solutions of the red pigment extracted from red human hair (broken line).

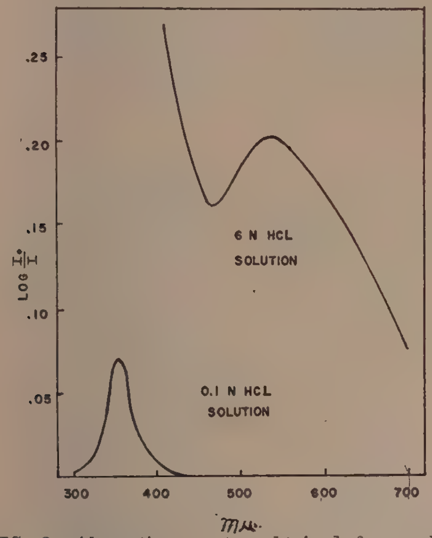

FIG. 2. Absorption spectra obtained from solutions of tryptophan and keratin treated together with .1 N and 6 N HCl when solutions containing the same concentrations of tryptophan and keratin, each of which has been treated separately with .1 N or 6 N acid, are used as standards.

treatment with 6 N acid has a spectrum showing the same points of maximum and minimum absorption as that of acid solutions of the reddish-purple phenolic iron pigment trichosiderin(7), which may be obtained from cortical granules of red human hair by heating with dilute mineral acids for short periods of time. Trichosiderin shows indicator properties and precipitates at neutrality(7,8). The tryptophan-keratin pigment contains no iron as indicated by negative thiocyanate and Prussian blue reactions, and the color is independent of pH.

The pigment isolated by chromatography has an amber color in 0.2 N NaOH. The color is independent of pH and the pigment does not precipitate at neutrality.

Summary. The soluble pigments produced when fat free nail keratin and tyrosine or tryptophan are boiled together with mineral acids differ from those produced when the keratin or amino acids are treated separately. The reaction involving tryptophan is enhanced by the simultaneous hydrolysis of keratin. Apparently keratin contributes some component(s) to the formation of pigment. The evidence favors the suggestion of Lee and Penrose that the red pigment obtained from human hair by the method of Arnow is not a natural pigment, but one produced from a colorless chromogen during the prolonged acid treatment. The pigment produced by the interaction of keratin and tryptophan in 6 N HCl shows an absorption spectrum similar to that of the pigment trichosiderin, but contains no iron. The similarity of the absorption spectra suggests that the organic portion of the natural pigment may be derived in part from tryptophan.

I am indebted to Dr. Herluf H. Strandskov, Dr. Hewson H. Swift and Dr. Morris Foster of the Department of Zoology, University of Chicago for their suggestions and criticism, to Dr. Foster for the amino acids used in this study and to Dr. Swift for his help with the spectrophotometric analyses.

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Hepatic Catabolism of Thyroid Hormone Studied by Radioactive Iodine in Splenic Autotransplants. (19598)

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(With the technical assistance of O. R. Czerwonka and A. L. Botkin.)

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Knowledge concerning the fate of thyroid hormone following its elaboration and discharge into the circulation is limited. Attempts to delineate the role of the liver in the catabolism of this hormone have yielded conflicting results. Early studies suggesting such a role (Asimoff and Estrin(1), Krayer(2)) used relatively large amounts of exogenous hormonal principles. More recently, Gross and Leblond(3,4), using intravenous injection of radioactively labelled thyroxine in animals, found significant localization in liver, gastrointestinal tract and feces. In their studies on the fate of ingested and infused thyroid hormonal principles in man, Albert and Keating (5), and Myant and Pochin(6) noted increased counting rates over the epigastrium, suggesting hepatic concentration of the material.

Gabe and Arvy(7) concluded from histological studies following pre-portal transplantation of the thyroid gland in rats that the liver completely removed thyroid hormone, whereas Bondy(8) concluded on the basis of protein bound stable iodine levels in a small number of similarly prepared experimental animals that the liver did not destroy and excrete the thyroid hormone to a significant extent. This report deals with the study in rats of radioactive iodine (I^{131}) uptake in thyroid tissue autotransplanted into the spleen and the subsequent level of plasma protein-bound I^{131} as an indirect measure of the removal by the interposed liver of endogenously labelled thyroid hormone.

Methods. Five groups of male albino Sprague-Dawley rats (150-250 g), maintained on stock Purina Laboratory diet were prepared, using intraperitoneal nembutal anesthesia. I. Thyroidectomized; II. Thyroidectomized, with thyroid gland implanted within muscle in the right axilla; III. Thyroidectomized with thyroid gland implanted within a splenic pouch; IV. Sham operated controls. These first 4 groups were studied, as outlined below, 24-39 days post operatively. A fifth group (Group V) of 11 animals, thyroidectomized with splenic implants, was followed for one year before sacrifice. Each animal was injected intraperitoneally at completion of the operative procedure with 10 cc of 1% calcium chloride in isotonic glucose solution. This solution also served as drinking water for 5-7 days, followed by tap water *ad lib*. thereafter. Weights were recorded periodically. Preliminary *in vivo* "scanning" studies were carried out at intervals. For this purpose 5 μc of carrier free I^{131}, in 1 cc of saline or Krebs-Henseleit buffer were injected intravenously (leg vein). At intervals after injection, external gamma counts were obtained by holding the region of the animal to be counted up to a lead collimated G-M tube (6 mm window), connected to a count-rate meter. For final studies of I^{131} uptake and plasma I^{131} levels, about 15 μc carrier free I^{131} were injected intraperitoneally. Blood was obtained at 2 and 24 hour intervals by direct cardiac puncture in the immobilized, unanesthetized animal. The protein-bound I^{131} level was determined by trichloracetic acid precipitation(9), expressed as PBI^{131} x 100/total plasma I^{131}. Uptake and conversion of I^{131} by transplanted tissue were determined by alkaline hydrolysis and fractionation(10). At the time of sacrifice a thorough search was made for residual thyroid tissue using a G-M tube for scanning purposes plus careful dissection; minute residual fragments in the neck could be detected in this way. The presence of adhesions between the spleen and abdominal wall or other organs was noted. The transplant and

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* Damon Runyon Fellow.

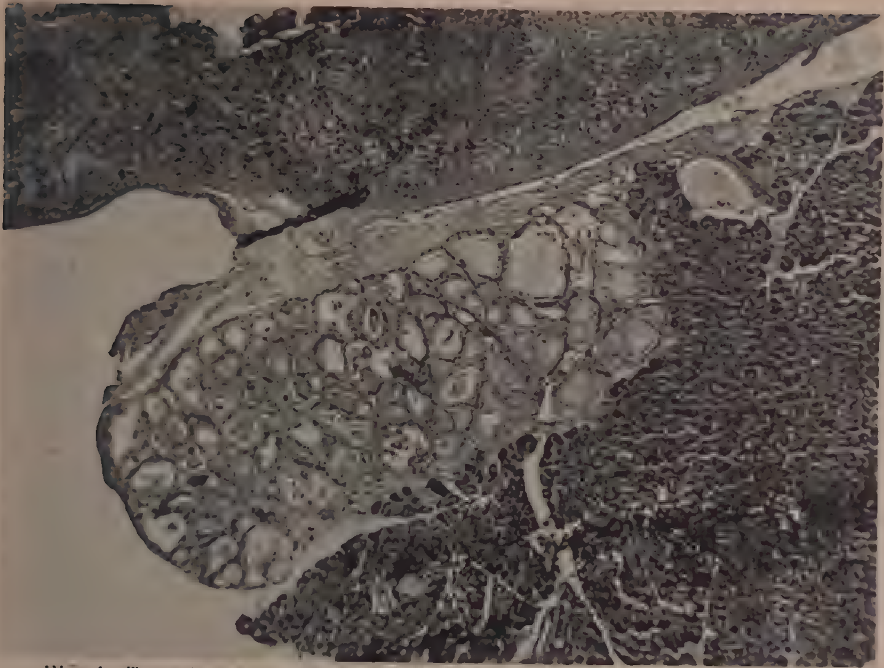

FIG. 1. Transplanted thyroid gland intimately associated with spleen and pancreas.

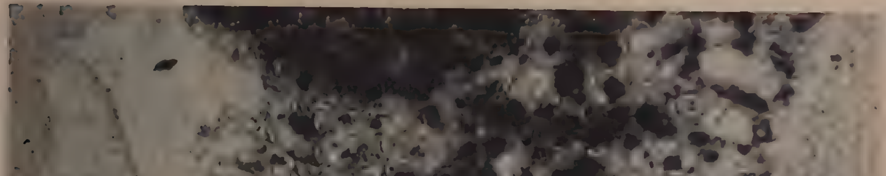

FIG. 2. Autoradiograph of intra-splenic thyroid gland (Eastman NTB plate, 50 μ).

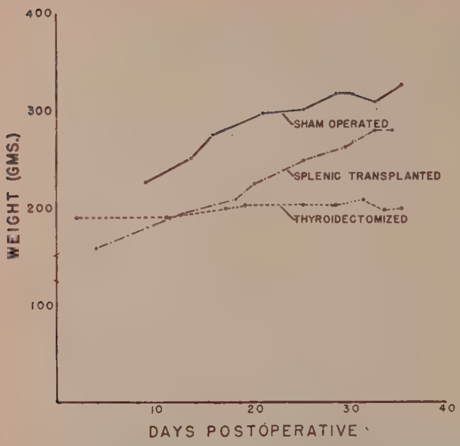

FIG. 3. Effect of thyroidectomy and splenic transplantation on avg wt curves.

residual neck thyroid tissue (if present) were removed for uptake and fractionation determinations and radioautography (Eastman NTB plates, 50 mμ thick). Other organs were fixed in Bouin's fixative for histological studies.

Results. Only one of 16 splenic transplanted animals died post-operatively. Viable, I^{131} concentrating tissue was found in 100% of the transplants, including those animals found to have residual thyroid tissue in the neck. There was no evidence of thyroid hyperplasia, hyperfunction or adenoma formation in the transplants (Fig. 1 and 2).

The thyroidectomized animals showed the classical "flattening" of the weight curve postoperatively whereas the curves for those with the splenic transplants showed a progressive weight gain comparable to those of the normal, sham-operated controls (Fig. 3). The splenic transplanted animals of Group V showed a progressive weight gain to values of 520-650 g at the end of one year.

Scanning of body areas by the use of a collimated G.M. tube and count-rate meter made it possible to confirm and follow at intervals in the same animal the presence of a functioning transplant and residual cervical thyroid tissue, if present. In normal animals there was a gradual, progressive rise in counts over the thyroid area for the first 24-48 hours; a comparable rise over the splenic area (attributed to contiguous structures, chiefly kidney) occurred during the first 4-6 hours, followed by a progressive decrease. In adequately thyroidectomized animals, the count over the thyroid area showed a sharp, progressive drop after 4 hours; residual tissue was suggested by a persistently high count beyond this time interval and confirmed, in each instance, by post-mortem examination. Similarly a persistently high level of radioactivity over the splenic area beyond 6 hours suggested a functioning transplant, confirmed at the time of examinations.

The percentage of I^{131} organically bound by transplanted thyroid 24 hours following uptake in 5 animals averaged 72.6 ± 10.9, a value in general agreement with those found in normal rat thyroids(10). The plasma PBI^{131} values at 24 hours following I^{131} injection are presented in Table I.

Residual cervical thyroid fragments were found in 5 operated animals. In 4 of these, the PBI^{131} values equalled or approached those of the controls (avg = 20.9 ± 5.9).

Discussion. The data presented are consistent with the interpretation that under the conditions described the liver removes or inactivates a fraction of the thyroid hormone delivered to it.

Failure to effect an athyreotic weight curve, to find evidence for compensatory thyroid hyperplasia or hyperfunction, or to obtain the low PBI^{131} values of thyroidectomized animals indicates that the liver does not completely remove the hormone. This contrasts with the interpretation of Gabe and Arvy(7) who based their conclusions of complete hepatic inactivation of thyroid hormone on histological studies of the pituitary, liver and adrenals of similar experimental animals but published no further data relative to the weight curves or metabolic status of their animals. Bondy (8) concluded that there was no significant hepatic destruction or excretion from data in 3 surviving animals with splenic transplants. He based this conclusion on control values of serum protein-bound stable iodine, but the possibility of residual thyroid tissue or adhesions by-passing the hepatic circulation was not ruled out. In agreement with the finding by Reinhardt(11) that about 50% of rats

TABLE I. Plasma PBI^{131} Levels 24 Hr Following Injection of I^{131}.

Group	No. animals	24 hr PBI^{131}
SHAM	4	29.9 ± 8.2
AXILLARY TRANSPL.*	3	23.2 ± 2.9
THYROIDECTOMIZED*	6	3.82 ± 1.6
SPLENIC TRANSPL.*	7	12.2 ± 5.4

* Without demonstrable residual cervical thyroid tissue.

subjected to surgical thyroidectomy still contain iodine-concentrating tissue, we found that even minute residual fragments resulted in PBI^{131} values at or approaching control levels.

In evaluating the data presented by Bondy it is further to be noted that since the PBI level is the resultant of 2 processes, *i.e.*, delivery to and removal from the vascular compartment, the interpretation of hormonal delivery on the basis of such levels rests on the assumption that the "peripheral utilization" of the hormone is not significantly altered. Knowledge concerning this latter parameter of thyroid function is limited. The data presented herein might thus be interpreted as representing an increased rate of hormonal utilization despite an unaltered delivery from the thyroid, through the hepatic circulation, to the systemic circulation. Further studies are necessary for a more complete clarification of the qualitative and quantitative role of the liver in the catabolism of thyroid hormone.

Summary. The role of the liver in the metabolism of endogenous thyroid hormone, labelled by I^{131} in splenic autotransplants has been studied in rats. Serial observations on the "functional take" of the transplants have been carried out. Data obtained by study of uptake and conversion of I^{131} and the resultant plasma levels of labelled protein-bound iodine indicate a partial removal or alteration of thyroid hormone by the liver under these experimental conditions.

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Composition of Urinary Calculi from Mink.* (19599)

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The occurrence of urinary calculi in mink is a problem frequently encountered on mink ranches. The incidence of urinary calculi is largely confined to 2 distinct periods of the year. In the spring calculi are found primarily in female mink during pregnancy or soon after whelping. The development of calculi before whelping is generally coincident with the presence of a large number of unborn kits. In the summer months calculi occur mainly in young male kits. In this country the occurrence of urinary calculi in mink appears to be limited primarily to the north central states. This regional difference in calculi formation may be related to the diet. In the Pacific Coast states, where there are relatively few cases of urinary calculi, the main constituents of the diet are fish and fish by-products. In the north central states, where the main component of the mink diet is horsemeat, there is a high incidence of urinary calculi. On some ranches calculi losses have been as high as 20% of the pregnant females. Although there has been an extensive qualitative chemical analysis of mink urinary calculi only a few calculi have been analyzed quantitatively. Sompolinsky(1) qualitatively analyzed 26 mink urinary calculi and classified 12 as pure magnesium ammonium phosphate, 13 as magnesium ammonium phosphate plus calcium, and one as magnesium ammonium phosphate plus calcium and urate. Smith and Hodson(2) qualitatively analyzed 6 urinary calculi and analyzed 5 calculi quantitatively. Of the calculi qualitatively analyzed 5 contained calcium, magnesium, phosphate and ammonia, while one contained only calcium, magnesium and phosphate. Their quantitative analyses of 5 mink urinary calculi indicated that the calculi were composed of magnesium ammonium phosphate and other magnesium phosphates. Calcium was present in small amounts in 2 of the 5 calculi.

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In our studies on the urinary calculi problem in mink variation in the type of calculi found seemed to warrant a more extensive quantitative study of their composition. The calculi analyzed were obtained from mink ranches in Wisconsin.

Results. In the analysis of the mink urinary calculi, phosphate was determined by the Fiske and Subbarow method(3). Calcium was determined by the Clark-Collip modification of the Kramer-Tisdall method(4), and magnesium by the method of Denis(5). Ammonia was determined by dissolving a portion of the calculi in acid, micro steam distillation of the ammonia after the addition of strong alkali into 2% boric acid and titration with standard acid. All analyses were carried out in duplicate.

TABLE I. Quantitative Analyses—Mink Urinary Calculi (Smooth, White and Hard Calculi).

Wt, g	% Ca	% Mg	% NH_4	% phosphorus
Pure $MgNH_4PO_4 \cdot 6H_2O$	0	9.9	7.3	12.6
1.44	2.2	8.7	—	11.8
1.59	2	8.5	6.5	11.6
1.91	1.9	8.2	6.5	12.8
1.63	1.6	8.9	6.3	12.1
1.48	1.4	9	6.3	12.2
.88	1.4	8.5	5.9	12.3
1.18	1.4	8.1	6.1	10.1
1.21	1.3	9.2	5.7	12.4
1.78	1.3	7.1	5.4	9.5
1.01	1.3	6.4	—	10.1
.49	.5	8.8	5.9	12.1
4.02	.2	9	5.2	12.5
.68	.1	9.1	6.5	12.2
4.31	.1	9.1	5	12
1.45	0	9.1	5.8	12.8
1.27	0	8.4	6	12.2

TABLE II. Quantitative Analyses—Mink Urinary Calculi (Rough, Colored and Friable Calculi).

Wt, g	% Ca	% Mg	% NH_4	% phosphorus
Pure $MgNH_4PO_4 \cdot 6H_2O$	0	9.9	7.3	12.6
.12	5.6	7.3	5.4	12.9
.06	4.1	6.9	—	10.8
.14	2.5	9	—	11.3
.44	2.3	8.9	5.8	11.7
.68	2	9.7	6.4	14.7
.12	1.5	9.5	6.6	12.1
.45	1.3	8	6.2	11.2
.84	1.3	8.4	6.4	11.8
.17	1.2	7.9	5.9	13.1
.56	1.1	8.9	6.3	12.1
.11	.9	9.1	—	11.7
.28	.3	9.2	6.2	11.8
.17	.2	9.8	6.3	12.6
.12	.1	9	6	11.8
.33	.1	8.9	6.1	12.4

From their gross appearance mink urinary calculi may be divided into 2 distinct types—the hard, smooth, white calculi, and the friable, rough, colored calculi. The size of the calculi vary from sand-like particles to stones weighing over 4 g. Chemical analyses of these 2 types of mink urinary calculi (Tables I and II) indicate that their composition is very similar. The main constituent of the calculi is magnesium ammonium phosphate hexahydrate ($MgNH_4PO_4 \cdot 6H_2O$). Small amounts of calcium phosphate and magnesium phosphate may also be present. The $MgHPO_4$ may have originated from the decomposition of $MgNH_4PO_4$. We have noted in our study of mink urinary calculi that the $MgNH_4PO_4$ constituent is unstable and is broken down into $MgHPO_4$ at temperatures less than 100°C. Some of the calculi analyzed were over 3 years old and partial decomposition of the $MgNH_4PO_4$ constituent may have occurred. The presence of organic matter was noted in all calculi analyzed. The results of these analyses agree with the analyses of Smith and Hodson(2).

Discussion. Since the main constituent of the mink urinary calculi is magnesium ammonium phosphate the question of the pH of mink urine arises. This type of calculi is known to form in an alkaline urine. Vermeulen, Grove, Goetz, Ragins, and Correll (6) have reported that there is a sharp drop in the solubility of magnesium ammonium phosphate over the range of pH 6.0 to pH 7.5. Kubin and Mason(7) found that the pH of mink urine varied over the pH range 6.8 to 7.5. These figures indicate that conditions may be favorable for the formation of this type of urinary calculi.

Summary. Thirty-one mink urinary calculi have been quantitatively analyzed for calcium, magnesium, ammonia and phosphorus. The results of the analyses indicate that the main constituent of the calculi is magnesium ammonium phosphate. Small amounts of calcium phosphate and magnesium phosphate may also be present.

We are indebted to Dr. G. R. Hartsough, Associated Fur Farms, New Holstein, Wisc., Capitol Fur Farm, Madison, Wisc., Pollack Mink Ranch, Whitewater, Wisc., and Red Cedar Mink Ranch, Cambridge, Wisc., for furnishing mink urinary calculi for these analyses.

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Comparative Measurements of Temperature in Liver, Lung, and Rectum of the Rabbit.* (19600)

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Several investigators have studied the temperature of either the lung or the liver (1-4), but no systematic study of liver and lung temperatures in the same animal has been recorded. Thus, Nedzel(1) reported that in dogs the liver temperature averages 0.2°C higher than the temperature of the aortic blood, and Tanaka(2) has shown that in man the lung temperature is 0.5-1.0°C lower than the rectal temperature. In the present study the temperatures within the liver, the lung, and the rectum have been measured simultaneously, or within a few seconds, in the normothermic rabbit.

Methods. Seven rabbits, which had been maintained on an ordinary diet, were lightly anesthetized with pentobarbital given intravenously. A No. 40 copper-constantan thermocouple sealed into a No. 20 hypodermic needle (with the thermocouple junction at the bevel of the needle) was inserted through the anterior abdominal wall on the right side, at a point 2-4 cm lateral to the tip of the xiphoid process, into the parenchyma of the liver. A similar thermocouple was inserted into the parenchyma of the lung through the chest wall, on either the right or the left side, pushing the needle through the 5th or 6th interspace anterolaterally. The current was read on a Rubicon galvanometer (Model 3401-H). The accuracy of the method was

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* We should like to express our appreciation to Dr. Balduin Lucké and to Dr. William Rashkind for their help.

TABLE I. Temperatures of Liver, Lung, and Rectum in 7 Rabbits.

Wt, kg	Avg liver temp.,* °C	Avg lung temp.,* °C	Avg rectal temp.,* °C	Avg thermal gradient in °C		
				Liver/Lung	Liver/Rectum	Lung/Rectum
5	38.73	38.51	38.70	+.22	+.03	—.19
2.2	39.10	38.97	38.81	+.13	+.29	+.16
2.6	40.51	40.38	40.17	+.13	+.34	+.21
2.5	39.01	38.75	38.70	+.26	+.31	+.05
2	39.00	38.86	38.80	+.14	+.20	+.06
2	39.28	39.11	38.85	+.17	+.43	+.26
2.7	39.48	39.28	39.07	+.20	+.41	+.21
Avg 2.7	39.30	39.12	39.01	+.18	+.29	+.11

* Each temperature recorded is an average of 7 readings.

±0.035°C. The rectal temperature was measured by a thermometer, with an accuracy of ±0.025°C. After the thermocouples and the thermometer were inserted, the temperatures of the 3 organs were recorded every 5 minutes for 30 minutes, each group of measurements requiring approximately 30 seconds. At the end of each experiment, the animal was killed and the position of the thermocouples determined. For our purpose a normothermic animal was considered to be one in which the rectal temperature did not vary more than ±0.3°C from the normal rectal temperature in the rabbit, which is 38.6-40.1°C(5). The ambient air temperature was 24 ± 2°C.

Results. Every animal was normothermic during the experimental period, and the thermocouples were actually in the substance of the liver and the lung. The results are summarized in Table I, which shows that the mean liver temperature exceeds the mean lung temperature in every animal, the average difference for all animals over the entire period of observations being 0.18°C. At no time was the lung temperature found to exceed the liver temperature. Also, in every animal, the liver temperature was found to exceed the rectal temperature in all observations, the overall average being 0.29°C. In all animals except No. 1, the mean lung temperature was higher than the rectal temperature, the average for the 7 animals being 0.11°C. In Rabbit No. 1, the lung temperature was consistently lower than the rectal temperature; and in Rabbit No. 5, the lung temperature was lower than the rectal in one observation, though the mean lung temperature was higher.

The temperature difference between the liver and lung and between the liver and rectum are statistically significant at the 1% level. The temperature difference between the lung and rectum is not statistically significant because of the measurements obtained in Rabbit No. 1. In the other 6 animals of the series, the lung-rectum difference also is significant.

Summary. In a series of 7 normothermic rabbits the liver temperature was consistently higher than either the lung or rectal temperatures. The lung temperature was usually, but not consistently, higher than the rectal temperature. The mean differences were as follows: a) The liver temperature averaged 0.18°C higher than the lung temperature. b) The liver temperature averaged 0.29°C higher than the rectal temperature. c) The lung temperature averaged 0.11°C higher than the rectal temperature.

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Effects of Desoxycorticosterone and Adrenocorticotrophic Hormone on Mercurial Diuresis.* (19601)

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Certain adrenal cortical hormones decrease renal sodium excretion while mercurial diuretics increase sodium excretion. Axelrod *et al.*(1) have claimed that in dogs the administration of a large dose of ACTH inhibits or markedly reduces a mercurial diuresis. If an antagonism between mercurials and the electrolyte-retaining adrenocortical hormones could be demonstrated this phenomenon would be of help in interpreting the action of the mercurial diuretics. It was thus of interest to investigate a possible antagonism between mercurial diuretics and the salt retaining adrenal hormones. Our present experiments include a study of the possible antagonism between the mercurial mersalyl and adrenocorticotrophic hormone (ACTH), desoxycorticosterone acetate (DOCA)† and desoxycorticosterone glycoside (DCG).‡

Methods. Dogs weighing between 12 and 22 kg were used in these experiments. In some of the dogs the action of the mercurial before and after desoxycorticosterone treatment was studied in the unanesthetized animal. Another series of experiments was conducted under pentobarbital anesthesia. The animals received intravenously a constant rate infusion of 0.86% sodium chloride or 3% glucose which contained adequate amounts of para-aminohippurate (PAH) and inulin or creatinine. Renal plasma flow was determined by the PAH clearance at low plasma concentrations while glomerular filtration was measured by the inulin or creatinine clearance. Sodium and potassium in plasma and urine were measured by means of an internal standard or a Beckman flame photometer. Chloride in plasma and urine were determined by the method of Van Slyke and Hiller(2). In the unanesthetized dogs blood specimens were obtained from a jugular vein while in the dogs under anesthesia arterial blood was obtained from the femoral artery by means of an inlying needle. The general procedures used in these experiments have been described previously(3). The mercurial used was mersalyl‡ which was given as the sodium salt or in combination with cysteine (1:1 molar ratio).

Results. Three groups of experiments were conducted. *Group 1.* Three unanesthetized female dogs received 3.2 to 3.6 cc of 3% glucose per m^2 of body surface per minute. After control periods of about 90 to 120 minutes, mersalyl and cysteine (1:1 molar ratio) were given in a dose of 8 mg per kg over a period of 2 to 3 minutes. The resulting diuresis was followed for about 2 hours after the injection of the mercurial. The same animal then received daily an intramuscular injection of desoxycorticosterone acetate (DOCA) in oil in a dose of 0.5 mg per kg. After 4 to 6 days of DOCA administration, a second diuresis experiment was conducted under the experimental conditions described above. The action of the mercurial was essentially the same before and after DOCA administration. In Table I the data obtained on 3 dogs have been summarized. In all 3 dogs there was no inhibition of either the mercurial-induced diuresis, natriuresis or chloruresis by prolonged DOCA administration. The changes in potassium excretion were variable. In 4 of the experiments the mercurial increased potassium excretion while in 2 experiments potassium excretion per minute was decreased by mersalyl. In all dogs urinary potassium concentration fell after the injection of the mercurial. The administration of DOCA over a 4- to 6-day period did not significantly modify the mercurial in-

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† Kindly supplied by Ciba Pharmaceutical Products, Summit, N. J.,

‡ Kindly supplied by Sterling Winthrop Research Institute, Rensselaer, N. Y.

TABLE I. Maximum Change in Sodium, Potassium, Chloride and Water Excretion Caused by Mersalyl before and after the Administration of DOCA in Unanesthetized Dogs (.5 mg/kg/Day). Mersalyl given intravenously 8 mg/kg.

Dog No.	Body wt, kg	Max increase in urine vol., cc/min		Max increase of excretion in: Na, mM/min		K, mM/min		Cl, mM/min		DOCA given
		Before DOCA	After DOCA	Before DOCA	After DOCA	Before DOCA	After DOCA	Before DOCA	After DOCA	
1.	14	+4.5	+3.6	+.45	+.58	+.03	+.04	+.50	+.65	5 days
2.	18	+4.9	+5.8	+.64	+.60	+.08	—.05	+.68	+.62	6 "
3.	12.5	+5.6	+6.5	+.72	+.80	—.02	+.04	+.80	+.80	4 "

duced net changes in potassium excretion.

Group 2. Anesthetized dogs received 3% glucose infusions at a rate of 3.5 to 4 cc/min./m^2 of body surface. The dogs received 3 mg/kg of DOCA dissolved in ethyl alcohol intravenously. About 90 to 120 minutes later 30 mg/kg of mersalyl and cysteine in a 1:1 molar ratio was given intravenously. In 3 dogs studied in this way there was a definite diuretic response to the mercurial which was about equal to that seen in dogs which had not received DOCA. In another group of 3 animals desoxycorticosterone glycoside was given in a dose of 5 mg/kg intravenously and about 30 to 45 minutes later 30 mg/kg of mersalyl with cysteine was given by the same route. Here again the mercurial diuresis was observed and was about the same in the DCG pretreated and the untreated control experiments. In one animal pretreated with DCG as above, mersalyl, in a dose of 2 mg/kg was given into the left renal artery. The injected left kidney showed a marked increase in water and sodium excretion while the right or control kidney was practically unchanged (Fig. 1). It must be concluded that neither DOCA nor DCG given acutely have the ability to inhibit the diuresis produced by mersalyl.

Group 3. Four anesthetized dogs received 14 to 17 cc/min./m^2 of isotonic saline intravenously. These dogs received ACTH (Wilson Lot 79951) in a dose of 70 to 100 units intramuscularly. Within 2 hours a definite reduction in urinary sodium concentration was produced by the ACTH in 3 of the 4 dogs and now mersalyl was given together with cysteine in a 1:1 molar ratio in a dose of 30 mg/kg. The high dosage of mercurial was used to produce maximal effects on the kidney (3). Here again the mercurial produced a rise in urinary sodium concentration and an increase in sodium and water excretion. The results of one of 4 typical experiments are shown in Fig. 2. This experiment shows that the mercurial can counteract the ACTH induced reduction in urinary sodium concentration and that BAL counteracted the mercurial effects. The increase in sodium excretion was determined graphically by plotting sodium filtered against sodium reabsorbed before and after the mercurial. The depression of sodium reabsorption produced by the mercurial was estimated by the shift of the straight line re-

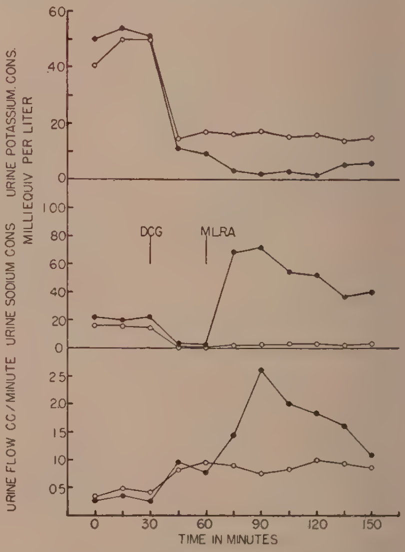

FIG. 1. Mersalyl diuresis in the desoxycorticosterone glycoside pretreated dog. Male dog 15.3 kg. Pentobarbital anesthesia. Ureters cannulated and excretion studies on each kidney. Infusion of 3.8 cc/min of a 3% glucose sol. Full circles = left kidney; open circles = right kidney. DCG: Intrav. inj. of 50 mg of desoxycorticosterone glycoside. MLRA: Inj. of 30 mg mersalyl into left renal artery for 2 min.

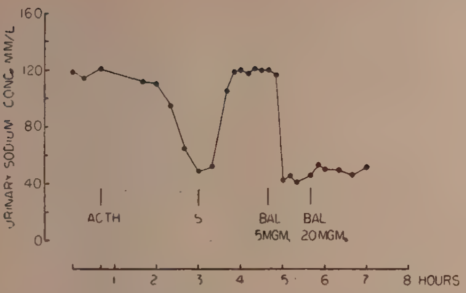

FIG. 2. Changes in urinary Na conc. following ACTH and mersalyl in the dog. Female 12.8 kg. Pentobarbital anesthesia. Isotonic saline infused 15.8 cc/min/m² body surface. ACTH: Intramusc. inj. 100 units ACTH (Wilson) S: 30 mg/kg of mersalyl with cysteine (1:1 molar ratio) intraven. BAL: 2,3 dimercaptopropanol intramusc. 5 and 20 mg/kg.

lating filtered to reabsorbed sodium. The details of this method have been described previously(3). By comparing the mercurial induced depression of sodium reabsorption with or without ACTH pretreatment the conclusion was drawn that ACTH in the dosage used and under our experimental conditions, did not alter appreciably the mercurial induced natriuresis and chloruresis (Table II).

Conclusion. Pretreatment of unanesthetized or anesthetized dogs with desoxycorticosterone acetate or desoxycorticosterone glycoside did not appreciably affect the diuresis induced by the mercurial mersalyl. In a similar manner ACTH in relatively large doses did not inhibit the mercurial diuresis. Our results indicate that a large dose of mersalyl actually produced a greater effect on tubular sodium reabsorption in the dogs receiving the ACTH. This increased excretion of sodium in the ACTH pretreated dogs is probably due to their initially lower urinary sodium concentration. The average values of the urinary sodium concentration at the time of the mersalyl injection was about 105 ± 12 for the control and 48 ± 6 milliequivalents per liter for the ACTH pretreated dogs. The peak increase in urinary sodium concentration following the mercurial was about 130 ± 4 and 135 ± 6 for the control and ACTH pretreated dogs respectively. It is of interest that the ACTH or DOCA induced reduction in urinary sodium concentration was readily counteracted by mersalyl. Mersalyl probably produced its renal effects by inhibiting some enzyme system essential for sodium reabsorption. Its ability to counteract the adrenocortical effects in the kidney may be due to an action of both these substances on the same enzyme system. However, mersalyl could conceivably produce the same results by acting on a sodium reabsorptive mechanism distal or proximal to the one acted on by the adrenocortical hormones. An action of mercury either distally or proximally could effectively mask the actions of the salt retaining

TABLE II. Depression of Sodium and Chloride Tubular Reabsorption Produced by Mersalyl on Control and ACTH Pretreated Dogs. A constant infusion of isotonic saline given intravenously at 15-17 cc/min in anesthetized dogs. Mersalyl given intravenously 30 mg/kg together with cysteine (1:1 molar ratio).

Exp. No.	Wt, kg	Depression of Na reabsorption	Depression of Cl reabsorption	
1	12.5	1.9	1.8	Controls
2	16	2.2	2.1	
3	14.8	1.9	2	
4	12	2	1.8	
5	17	2	2	
6	15.5	1.5	1.5	
7	15	1.4	1.5	
Avg		1.83±.36	1.81±.26	
1	14.8	2.6	2.5	ACTH (Wilson) given intramusc. 75-125 units about 2 to 3 hr before mersalyl was inj.
2	12.9	2.5	2.6	
3	16.5	2	2.2	
4	14	1.8	1.9	
Avg		2.22±.39	2.30±.32	

adrenal hormones without necessarily acting directly on the same renal tubular locus affected by the adrenocortical hormones. The results obtained with ACTH are different from those reported by Axelrod and MacLeod(1). Since no details regarding procedures and methods are available at this time, it is not possible to explain the differences in our results and those of Axelrod and MacLeod.

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Pyruvate Metabolism and Assessment of Semen Quality. (19602)

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During a study of the metabolism of pyruvate in mammary tissue it was observed that 2:4-dinitrophenol (DNP) stimulates the respiration without increasing the rate of utilization of the substrate, thus causing its more complete oxidation(1). Preliminary experiments in which the effect of DNP on the metabolism of pyruvate in spermatozoa was tested produced somewhat similar results(2). Since DNP is known to inhibit synthetic reactions(3) it was thought that it might be a useful reagent for an assessment of the quality of spermatozoa based on their synthetic power.

Experimental procedure. Washed bull spermatozoa(4) were suspended in phosphate saline(5), and incubated at 37° in the Warburg apparatus a) without addition, b) with pyruvate (0.005-0.01 M) and c) with pyruvate + DNP (10^{-4} M). All flasks also contained fumarate (0.002 M). The oxygen consumption was followed for one to 2 hours. Pyruvate and lactic acid were determined by chemical methods(6,7). Since pyruvate is rapidly reduced to lactic acid by spermatozoa under aerobic conditions(2), the amount of pyruvate metabolized by oxidative reactions is given by the difference between the amounts of pyruvate disappearing and lactic acid formed and is designated as "net pyruvate"(8).

Results. In early experiments in which semen from selected bulls of high fertility was used, the respiratory rate in the presence of pyruvate was often accelerated by DNP(2), but in samples from other equally fertile bulls, it remained unaltered or was slightly depressed. In all these experiments DNP increased the ratio O_2 consumed/net pyruvate. The fact that the observed ratios were relatively high and in the presence of DNP often exceeded the theoretical 2.5 suggested that the endogenous respiration was incompletely suppressed.

On addition of fluoride (0.02 M) the endogenous respiration of spermatozoa was reduced to a low level; although the level of respiration in the presence of pyruvate was also depressed, DNP now caused marked stimulation of the oxygen consumption in the presence of the substrate also in those samples in which, without fluoride, it was inhibitory (Table I). DNP did not stimulate the respiration in the absence of added substrates, or in the presence of small amounts of endogenous lactate (less than 5×10^{-4} M).

When the respiratory response to pyruvate and DNP of a larger number of samples was examined in the presence of fluoride, 5 groups could be distinguished. *A.* The respiratory rate in the presence of pyruvate was low, very little above the endogenous level; DNP caused large increases in O_2 consumption, often exceeding 100%. *B.* The rate of respiration in pyruvate was about twice the endogenous level and DNP caused little or no further increase. *C.* The respiratory rate in pyruvate was inter-

TABLE I. Effect of Pyruvate, Fluoride and 2:4-Dinitrophenol of Respiration of Spermatozoa. Washed bull spermatozoa incubated in phosphate saline at 37°; gas, air. Fumarate (.002 M) in all flasks. Pyruvate, .01 M; fluoride, .02 M; DNP, 10^{-4} M. Volume, 4 ml. Sperm count/flask: Exp. I, 9.7×10^8; Exp. II, 7.92×10^8; Exp. III, 12.6×10^8. Incubation period: I, 45 min; II, 70 min; III, 120 min.

	Z_{O_2} (μl/10^8 spermatozoa/hr)		
Additions	I	II	III
0	−18	−11.7	− 6.8
Pyruvate	−25.9	−21	−17.5
" , DNP	−25.8	−19.2	−23.1
Fluoride	− 4.2	− 4.2	− 4.8
" , pyruvate	− 6	− 5.2	− 7.6
" " , DNP	−11.3	−11.8	−15.6

TABLE II.* Metabolic Response of Bull Spermatozoa in Relation to the Fertility of the Donors.

			Type of metabolic response		
Avg fertility of bulls (as % nonreturns at 16 wk after insemination)	No. of bulls	No. of samples tested	Good (A)	Intermediate (C)	Poor (B,D,E)
>65	3	11	9	2	0
55-65	8	22	7	8	7
<55	5	27	4	2	21
Unknown	3	10	2	1	7
Total	19	70	22	13	35

* We wish to thank Miss Zena D. Hosking for the statistical analysis of the data.

At least 3 samples of semen from each bull were tested, except in 4 cases.

$\chi^2 = 27.4$ (4 d.f.) $P < .001$. If the data are grouped within two classes only—above or below 55, then $\chi^2 = 19.1$ (2 d.f.) $P < .001$.

mediate between the endogenous and the pyruvate-plus-DNP levels. *D.* Both pyruvate and pyruvate-plus-DNP caused only small increases of respiration above the normal endogenous level. *E.* In a few experiments the endogenous respiration was exceptionally low and the addition of substrate and DNP was without effect.

All samples were graded according to the usual criteria (sperm count and motility) at the time of collection, and the average fertility of each donor was computed from its breeding record in artificial insemination during the past 12 months. No connection could be found between the type of respiratory response and the motility of the spermatozoa, which in most cases was good. However, a correlation between the metabolic response of the individual samples and the known fertility of the donors is apparent from Table II.

Chemical estimation of the metabolites involved showed that spermatozoa of type A metabolized pyruvate with a low O_2/net pyruvate ratio (less than 1) which was increased by DNP to 2.0-2.5, a result expected in tissues of high synthetic capacity(1). Samples of type B, despite their high respiratory activity, revealed their low synthetic power by oxidizing pyruvate to completion in the absence of DNP. Spermatozoa of type C showed intermediate O_2/net pyruvate ratios. In samples of type D, pyruvate was metabolized at slow rates and oxidized to completion (Table III).

Conclusions. 1. The results described above suggest that manometric measurements of respiration in a system comprising fluoride, pyruvate and DNP may provide a measure of the metabolic activity and give an indication of the potential fertilizing capacity of spermatozoa. Although other factors outside the scope of the manometric test play a large part in determining fertility, it seems to be generally recognized that the metabolic vigor of the spermatozoa is an important property in relation to their capacity for survival and fertilization(9). It should be pointed out that, since the observed metabolic rates represent the mean of the metabolic activities of a large number of spermatozoa, samples classified as poor may yet contain a small proportion of spermatozoa of good quality and under favorable conditions effect fertilization. In fact none of the bulls tested was completely sterile, but those of an average fertility of less than 55% as computed for Table I, are considered of little value as donors of semen for artificial insemination. 2. Although the correlation between the respiratory response and the fertility of the sperm samples in the field could not be tested on a larger scale than that reported, the test is put forward at this stage to make it available for more extensive trials by other workers. Present experience suggests that the test may prove its usefulness in helping to detect bulls which produce semen of exceptionally high quality (type A), and also

TABLE III. Metabolism of Pyruvate in Spermatozoa. Washed bull spermatozoa incubated in phosphate saline at 37°; gas, air. Fumarate (.002 M) and fluoride (.02 M) in all flasks. Pyruvate (.01 M) unless otherwise stated. DNP, 10^{-4} M. Volume, 4 ml.

Type of metabolic response	Incubation period, min	Sperm count /flask × 10^8	Additions	Z (μl/10^8 spermatozoa/hr)				Ratio O_2/net pyruvate
				O_2	Pyruvate	Lactic acid	Net pyruvate	
A	70	7.92	0	− 4.2	—	—	—	—
			Pyruvate (.005 M)	− 5.2	−25.3	+16.3	−9	.6
			" " , DNP	−11.8	− 7.7	+ 2.4	−5.3	2.2
B	90	10.2	0	− 8.6	—	—	—	—
			Pyruvate	−14.1	−30.5	+23.4	−7.1	2
			" , DNP	−16	−12.5	+ 4.4	−8.1	2
C	90	11.7	0	− 4.9	—	—	—	—
			Pyruvate	−11.5	−38.7	+29.6	−9.1	1.3
			" , DNP	−14.9	− 7.4	+ 1.2	−6.2	2.4
D	120	9.1	0	− 6	—	—	—	—
			Pyruvate	− 9.1	−12.8	+ 9	−3.8	2.4
			" , DNP	− 8.2	− 7	+ 3.6	−3.4	2.4

semen of poor fertilizing capacity (types B and D) which, because of its good appearance and motility, might otherwise be considered satisfactory. The test may also be used for a periodic check on the quality of semen produced by bulls of fluctuating fertility. 3. Fructose, glucose, or lactate are not suitable as substrates since their utilization is in all cases increased by DNP.

Summary. Bull spermatozoa were graded according to their metabolic behavior on incubation with pyruvate, fluoride and 2:4-dinitrophenol. A correlation was shown between the type of response of the samples *in vitro* and the fertility of the donors in the artificial insemination of cattle.

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Method for Determination of Vitamin A in Stool.* (19603)

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Studies conducted in this laboratory on the metabolism of vit. A required a method for the quantitative determination of the concentration of this vitamin in stool. A search of the literature failed to reveal a satisfactory method. The procedure reported by Wendt (1) involves drying the stool with anhydrous sodium sulfate and subsequent extraction of vit. A with alcohol. This procedure is time consuming and may destroy some of the vitamin. Wendt did not report the degree of recovery of vit. A by his method. The following quantitative method for determination of vit. A in stool was therefore developed.

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* This study was aided by a grant from the National Vitamin Foundation.

The method depends upon preparation of a uniform suspension utilizing a magnetic stirrer. Samples of the suspension are then digested with alkali. A mixture of xylene and kerosene is used to extract the vit. A from the digested suspension again using the magnetic stirrer. The extracted vit. A is then determined by ultraviolet destruction as in the Bessey *et al.* method(2).

Method. The entire stool specimen is weighed and transferred into an Erlenmeyer flask. A weighed amount of 6% alcoholic potassium hydroxide is added. The amount of this potassium hydroxide to be added is estimated by experience according to the consistency of the original stool. If the stool is watery, considerably less of the potassium hydroxide solution is needed than if it is more solid. This amount may vary from one-third to an equal quantity of the weight of the stool. A magnetic stirring bar is placed into the flask and the whole placed on the magnetic stirrer until there is a uniform suspension. Between 2 to 3 g of this suspension are then weighed into a test tube of approximately 20 ml size. The volume in the test tube is made up to about 5 ml by addition of 6% alcoholic potassium hydroxide. The test tube is then covered with a glass marble and placed in a water bath at 70°C for 6 to 8 hours. (If the digestion cannot be finished the same day, the test tube may be kept in a refrigerator overnight.) The digested mixture is then filtered (Whatman No. 40 filter paper) by gravity into a 10 ml volumetric flask; the filter is washed with 6% alcoholic potassium hydroxide solution until the volume in the volumetric flask is 10 ml. To 2 ml of this alkaline filtrate in a 50 ml Erlenmeyer flask, add 2 ml of a xylene-kerosene (1:1) mixture. The magnetic bar is placed into this flask and the mixture is agitated vigorously for 10 minutes. The mixture is then allowed to stand until the layers separate. From the supernatant xylene-kerosene extract, 2 aliquots of 0.08 ml are drawn. The extinction coefficients of the samples are read in a Model DU Beckman spectrophotometer at 328 mμ; the samples are then irradiated and the extinction coefficients are again read at 328 mμ in the manner described by Bessey *et al.*(2). The average of the results obtained from the 2 aliquots is reported. In case the vit. A content of the stool proves to be too low to read in the spectrophotometer, the determination should be repeated by extracting 2 ml of the alkaline filtrate with 1 ml of the xylene-kerosene mixture as stated above.

Calculation. The vit. A values are obtained by the following calculation: $\frac{E_1 - E_2}{v - w} \times 637 =$ μg vit. A in 1 g stool, where E_1 is the extinction coefficient at 328 mμ before irradiation, E_2 is the extinction coefficient after irradiation, v is the quotient of 100 divided by the volume to which the filtrate was made up after saponification, w is the weight of stool sample in g; 637 is Bessey's factor.

Results. Suspensions of 10 different stools with alkali were prepared as in the first step described above. Two samples were removed from each suspension and analyzed for vit. A. To the remainder, a known amount of vit. A was added. The suspension with added vit. A† was stirred thoroughly again with the magnetic stirrer for at least 10 minutes. Two more samples were removed and the vit. A concentration was determined on each sample.

Table I shows the duplication of results which may be expected. The mean percentage difference between 2 determinations on the same stool suspension was 9.1% with a range of 0 to 18% (stand. dev. 5.2%). Table I also shows the per cent of recovery of known amounts of added vit. A. These recovery tests show that the mean per cent recovery is 6.25 (range 1 to 18) and that the standard error of a single determination (stand. dev.) is 7.5%.

It was found that if stool samples are kept in tightly closed "ointment type" jars in a refrigerator, the vit. A. concentrations do not change for at least 72 hours.

Discussion. The first requirement in a method for the accurate determination of vit. A concentration in stool is the preparation of a homogeneous stool sample. Drying of the

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† The vitamins used in this study were kindly supplied by the Hoffmann-La Roche Co., Nutley, N. J.

TABLE I. Recovery of Added Vitamin A from Stool.

	Vit. A, μg/g stool					
Human subject	Before addition of vit. A	Mean	Vit. A added	After addition of vit. A	Mean	% recovery of vit. A
T.	2.05	2.17	3.5	6.30	6.19	109.1
	2.28			6.12		
V.	1.80	1.78	2.26	4.00	4.00	101
	1.76			4.00		
C.	1.59	1.62	2.81	4.15	4.36	101.4
	1.65			4.6		
J.S.	5.2	5.5	22.2	30.8	32.7	118
	5.9			34.5		
P.	5.8	6.35	16.7	23.9	25.4	110
	6.9			26.9		
H.	5.8	5.45	7.85	14.3	12.5	99
	5.1			13.6		
F.T.	.75	.83	5.00	5.2	5.6	96
	.90			5.9		
J.C.	2.26	2.13	28.6	32.4	31.1	101.3
	2.00			29.8		
M.	0	0	14.9	16.1	16.9	113.4
	0			17.6		
S.M.	3.3	3.1	15.6	17.3	18.1	96.7
	2.9			18.8		

stool and sampling of the dry powdered mixture is not satisfactory since drying of stool requires considerable time, and since vit. A is not stable to heat and oxygen. Preparation of a homogeneous sample from fresh stool was a difficult problem until it was solved by using a magnetic stirrer as described above. The size of the stirring magnet can be varied according to the amount of stool so that even small quantities may be used. Thus well blended mixtures can be prepared from which uniform samples can be taken.

Summary. 1. A method is described for the quantitative determination of vit. A in stool. 2. Alcoholic alkali is added to fresh stool and a uniform mixture prepared by using a magnetic stirrer. The method of extraction is described in detail. The standard percentage deviation of the difference between 2 determinations on the same stool was shown to be 5.2% and the standard deviation of the per cent recovery of added vit. A is 7.5%.

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Protogen and its Relation to Ketoacids in Metabolism of Tetrahymena.[*] (19604)

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The function of protogen (thioctic acid, Factor II, acetate factor, pyruvate oxidation factor, α-lipoic acid(1-6)) in the metabolism of lactic acid bacteria(4,5,7) appears to be concerned solely with the oxidation of pyruvate with the simultaneous production of acetate. In the case of *Tetrahymena geleii*, however, it has been shown that acetate

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[*] Aided by a grant from the Research Corporation.

[†] Generously supplied by Dr. E. L. R. Stokstad of the Lederle Laboratories.

merely spares and does not replace protogen (8).

Cultures of *Tetrahymena* grown in acetate-free protogen deficient media showed only limited growth followed by death and accompanied by a drop in pH (to 4.0 or below). A similar observation has recently been made by Slater(9) on another strain of this organism. The object of this report is to identify the acids accumulated in such cultures.

Methods. The organism used was *Tetrahymena geleii* strain W. Cultures were grown in Medium A80(10). The concentrations of protogen† and acetate were varied in different experiments. The effect of the addition of various acids of the tricarboxylic acid cycle and of various fatty acids was also studied. The presence of carbonyl compounds in the cultures was demonstrated by preparation of the 2,4-dinitrophenyl hydrazones by the usual procedures after deproteinization with trichloracetic acid. The dinitrophenyl hydrazones (DNPH) were extracted with either ethyl acetate, if to be used for paper chromatography, or with xylene. Tentative identification of these compounds was made on paper chromatograms by comparing them to the DNPH of commercial samples of pyruvic, α-ketoglutaric, oxalacetic(11) and acetoacetic acids, using the *n*-butanol-ammonia solvent of Cavallini *et al.*(12). The materials were further characterized by differential solubility (13) in xylene and by their absorption spectra.

Results. Table I shows the R_f values obtained from paper chromatograms of the DNPH of various known compounds as well as those for a culture deficient in protogen. It will be noted that all of the known compounds, except acetoacetate, give 2 spots in this system(14). Deficient cultures give spots corresponding closely to those of pyruvic acid DNPH as well as spots which might be those of either oxalacetic or α-ketoglutaric acid DNPH. Other solvents failed to differentiate these 2 compounds. There is in addition another spot moving close to the solvent front, as does that of acetoacetic acid DNPH. No attempt has been made to identify these 2 compounds, however.

In order to determine the nature of the material showing spots with R_f values of 0.07 and 0.72, the DNPH of deficient cultures were extracted with xylene to remove the DNPH of pyruvic acid. The residue was then made alkaline with 2 N NaOH and the absorption spectrum determined. As may be seen from Table II, the ratios of the absorptions at 430 mμ and 500 mμ show better agreement between the material obtained from the cultures and α-ketoglutaric acid than between the former and oxalacetic acid. The same is true of the absorption maximum. It is probable that more than one compound is present in the residual fraction.

Relative concentrations of the xylene-soluble and the xylene-insoluble fractions of cultures containing varying amounts of protogen with or without added acetate or fatty acid were also followed. Fig. 1 and 2 show the relationship between growth, pH and the accumulation of carbonyl compounds in such cultures. It is to be noted that in cultures containing no acetate the greatest rise in the non-pyruvic acid carbonyl compounds occurs at protogen concentrations in which the pyruvic acid has begun to decline (Fig. 1), while in cultures containing acetate or fatty acid (pentadecylate, Fig. 2) the 2 are more or less parallel. All carbonyl compounds fall to a low level as the amount of protogen approaches an amount adequate for growth (1 unit per ml). Omission of glucose from

TABLE I. R_f Values Obtained from Paper Chromatograms of the 2,4-dinitrophenyl Hydrazones of Keto Acids. (Solvent: Butanol-ammonia; ascending.)

Acids	R_f values
Pyruvic acid	.36; .59
Oxalacetic "	.10; .70
α-ketoglutaric "	.09; .70
Acetoacetic "	.96
Protogen deficient culture	.07; .38; .58; .72; .92

TABLE II. Spectral Absorption Data of the 2,4-dinitrophenyl Hydrazones of Keto Acids.

Acids	Ratio, 430 mμ:500 mμ	Max mμ
Oxalacetic acid	1.39	442
Pyruvic "	1.55	438
α-ketoglutaric "	1.98	428
Protogen deficient culture Non-pyruvic acid fraction	1.79	428–430

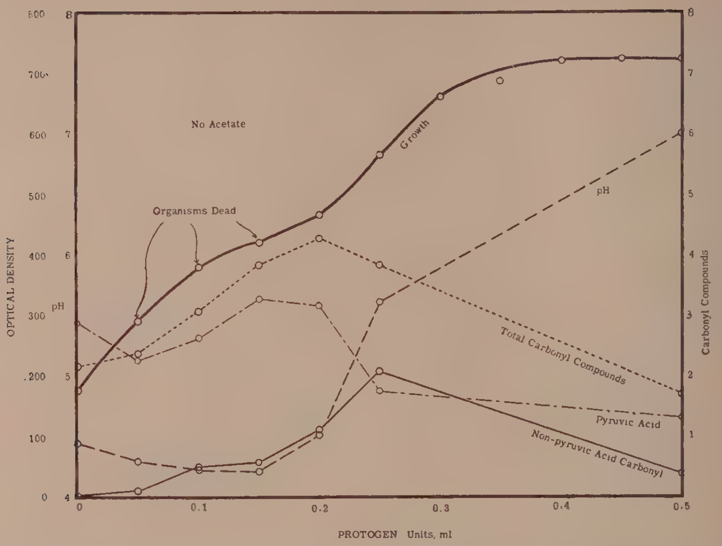

FIG. 1. Relationship between growth, final pH of medium and accumulation of carbonyl compounds as protogen content of medium is increased. Growth (optical density) and pH on left hand scale. Concentration of carbonyl compounds in arbitrary units on right hand scale.

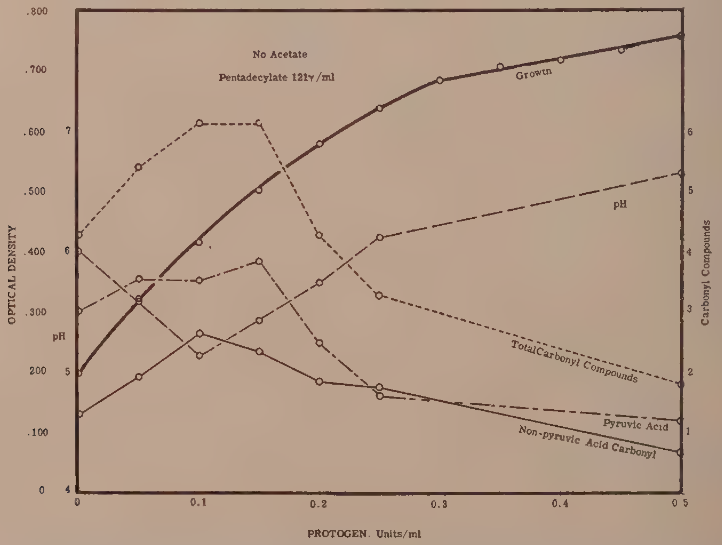

FIG. 2. Relationship between growth, final pH of medium and accumulation of carbonyl compounds as protogen content of medium is increased. Pentadecylate present, .5 micromole per ml. Growth (optical density) and pH on left hand scale. Concentration of carbonyl compounds on right hand scale (arbitrary units).

the medium prevents the accumulation of acid, while the omission of glutamic acid does not.

Since the organisms do not die when acetate is present in the medium, even though acids are accumulated to approximately the same extent in its presence or in its absence, a part of the effect of acetate must be attributed to its buffering capacity. A part of the "buffering effect" may be due to the contribution of Na^+ to the medium as the acetate is oxidized. A similar effect is obtained if the pH of the medium is adjusted to 7.6 before inoculation and the organisms are not killed by acid accumulation. The fact that fatty acids (valerate through stearate) also have a sparing effect on protogen even though present in amounts too small to contribute significant amounts of Na^+, indicates that acetate has a real effect in and of itself, since fatty acids may be considered to be a source of "active acetate." In fact fatty acids are a more efficient source of "acetate" than is acetate itself, since maximal effects are obtained with 0.5 micromole of fatty acids of 5 or more carbons while approximately 4.0 micromoles of sodium acetate are required. Pentadecylate was chosen to replace acetate for use in these studies because the lower fatty acids (propionate and butyrate) are relatively ineffective in replacing acetate, those of intermediate chain length (caproate through laurate) cause considerable inhibition of growth and those of longer chain length (palmitate and stearate) are too insoluble for practical use. Valerate and pentadecylate represent compromises between these factors. The Krebs cycle acids tested (citric, *cis*-aconitic, succinic, malic, fumaric and oxalacetic, added as their sodium salts) were effective in preventing death of the organisms only in so far as they contribute Na^+, since the sodium salts of such unnatural materials as *trans*-aconitic acid, maleic or malonic acids were equally effective.

Discussion. Although the acids accumulated by cultures of *Tetrahymena* deficient in protogen have been identified only tentatively as consisting mainly of pyruvic and α-ketoglutaric acids, the presence of the former was suspected from the known function of protogen in the oxidation of pyruvic acid by lactic acid bacteria. The accumulation of a second acid only as pyruvate disappears and its increased concentration in the presence of acetate or a source of "acetate" suggests that the acid is a Krebs cycle intermediate requiring acetate for its formation. Since the material contains a carbonyl group, oxalsuccinic, α-ketoglutaric and oxalacetic acids are possibilities. Of these α-ketoglutaric appears to be the most probable, not only because of its greater stability, but because of the similarity of the reactions involved in its oxidative decarboxylation to those with pyruvic acid. Both reactions are known to require thiamine pyrophosphate, diphosphopyridine nucleotide and Coenzyme A, and both reactions yield a large amount of energy. This appears not to be true of the decarboxylation of oxalacetic acid. That the α-ketoglutaric acid does not represent merely a deamination product of glutamic acid is demonstrated by its presence in cultures grown in the absence of added glutamic acid and the failure of the cultures grown in the absence of glucose to accumulate acid.

In view of the ability of lactic acid bacteria to utilize acetate in the absence of protogen (acetate factor), it was hoped that a similar substitution could be made in the case of *Tetrahymena*. Consequently a mixture of acetate and succinate was supplied in cultures lacking protogen. No growth occurred. The failure to grow on this mixture may be only an indication of the impermeability of the cells to succinic acid as suggested by Seaman and Houlihan(15). This explanation seems rather improbable, however, in view of the fact that these organisms have mouths and that material is taken into food vacuoles. It therefore appears probable that protogen also functions at some point in the metabolism of *Tetrahymena* other than in the production of acetic and succinic acids.

Summary. Tetrahymena geleii strain W was grown in media in which the concentrations of protogen and of acetate were varied. The effect of additions of Krebs cycle intermediates and of fatty acids to such media was studied. In deficient media the organisms grow to a limited extent and then die. Death is due to the acidity of the medium. The acids accumulated have been tentatively identified as consisting mainly of pyruvic and α-ketoglutaric acids using paper chromatography and differential solubility of the 2,4-dinitrophenyl hydrazones. α-ketoglutaric acid accumulates at the expense of pyruvic acid in the absence of added acetate. In the presence of acetate or of higher fatty acids pyruvic acid and α-ketoglutaric acid accumulate in about equal amounts. A mixture of succinate and acetate does not replace protogen. A function other than the oxidation of pyruvic and α-ketoglutaric acids in the metabolism of *Tetrahymena* is postulated.

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Effect of Hyaluronidase on Edema Following Experimental Cold Injury.* (19605)

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The formation of edema following experimental frostbite is accompanied by rising interstitial fluid pressure and distortion of tissues which may cause obstruction of capillaries(1,3,5). The resulting stasis with limited exchange of metabolites would suggest that the accumulating edema is a contributing factor in the resulting tissue loss(3,5). Several attempts therefore have been made to control the fluid loss from the blood stream into the injured tissues(2,3,8,10,11,12). In our experiments we attempted to achieve a similar objective by use of hyaluronidase. This enzyme reduces the resistance of tissues to fluid absorption and increases tissue permeability(4,7). We have investigated whether facilitation of absorption of edema fluid with corresponding decrease in swelling would influence the appearance and extent of the resulting gangrene.

Material and methods. Six adult albino rabbits weighing 2450 to 2850 g were used. All animals were anesthetized with pentobarbital sodium intraperitoneally. Both depilated hind legs were immersed for 4 minutes in a medium consisting of ethylene glycol, alcohol and water, previously cooled to a temperature of —35°C to —37°C. The exposed extremities were allowed to thaw in air at room temperature. Hyaluronidase (Schering) was injected subcutaneously into the edematous tissues of one of the injured extremities using aseptic technic. The other extremity served as a control. One hundred and fifty turbidity-reducing units (TRU) of the enzyme, dissolved in 1 cc of distilled water, were injected in 3 different places on both the medial and lateral surfaces of the extremity. Thus a total dose of 300 TRU was injected into one extremity in 24 hours. The first injection was made 3 hours after exposure to cold, the other 24 hours later. The changes in volume of both extremities representing actual alterations in edema formation were determined by measuring the amount of water displaced during immersion of each extremity to a standard

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* This work was supported by a grant-in-aid from the Defence Research Board of Canada.

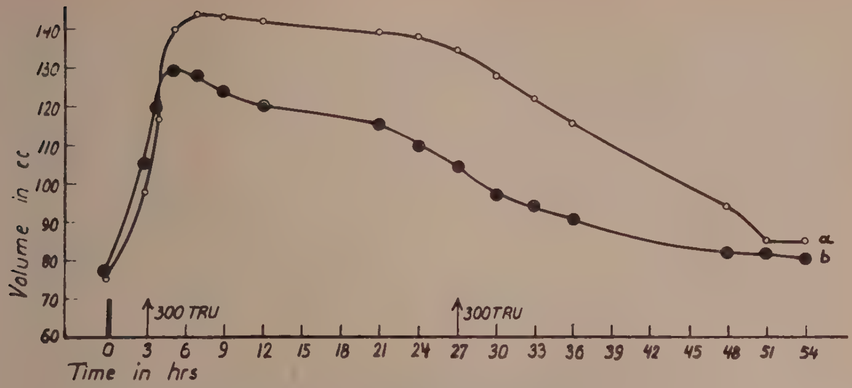

FIG. 1. Comparison of volume changes in rabbit legs after exposure to severe cold (I). The lines indicate the mean values in 6 extremities treated with hyaluronidase (b) and in the controls (a).

mark. The measurements were taken prior to exposure to cold, before each injection of hyaluronidase and at intervals of 3 hours.

Observations. The changes in volumes of both exposed extremities indicating the actual alterations in edema formations during the experimental period are presented in Fig. 1. The lines demonstrate the mean values of measured leg volumes of all rabbits. The upper line a) shows the mean values of control frozen extremities, while the lower b) that of frozen legs treated with hyaluronidase.

The thawing of the extremities was followed by rapid and extensive swelling expressed by increase in volume by about 53%, 3 hours after injury. Two hours after injection of hyaluronidase there is no further swelling in the treated extremities b), while the controls show a progressive augmentation in volume by about 87% a). During the following 22 hours the volume of treated extremities declines progressively b), whereas that of injured but not injected legs remains constant a). Further diminution in volume of treated extremities can be observed in the 24 following hours after the second injection of the enzyme while the controls showed a lesser decrease in volume. Fifty-four hours after injury there are no significant differences in the volumes of treated and control extremities.

Wet gangrene developed in all animals about 50 hours after injury in both treated and control extremities simultaneously. There were no differences in the extent of the gangrene in both legs.

The routine histological examinations of the post mortem specimens taken from treated and untreated extremities showed an extensive necrosis of injured tissues. There was less edema in subcutaneous tissue and interstitial spaces in specimens taken from hyaluronidase treated extremities.

Discussion. The incorporation of hyaluronidase into the edematous tissues previously injured by cold prevented further swelling of the treated extremity and caused a progressive and significant decline in volume as compared to controls. The cessation in edema formation and the evacuation of accumulated extracellular fluid may be due to reduction of the viscosity of the hyaluronic acid gel with a resultant lessening in resistance to fluid diffusion (6,7,9). The accelerated reabsorption of accumulated edema fluid results in a marked decrease in volume of the swollen extremity.

The removal of edema did not prevent the development of gangrene which appeared simultaneously in both the treated and control extremities in all animals. There were no differences in the extent of final tissue loss nor in the microscopical appearance of the cells damaged by cold. The failure to prevent or to restrict the formation of gangrene by the use of hyaluronidase in our experiments is in contrast to the results obtained by mechanical control of edema. Crismon and Fuhrman(3) reported that application of casts and elastic pressure dressings before and after thawing restricted significantly the amount of gangrene. The most beneficial effects were obtained by using these methods combined with rapid rewarming. These results were not confirmed by other investigators. Lange and Boyd(8), using pressure dressings with and without heparinization, did not observe any beneficial effect. Quintanilla *et al.*(10) stated that pressure dressings and plaster casts were ineffective. Shumacker and his associates (11) demonstrated, in experiments conducted with precise controls, that plaster casts left in place for 10 days, as recommended by Crismon and Fuhrman, were without benefit.

The problem whether the accumulating edema following cold injury is a major contributing factor to tissue loss, cannot be definitely determined on the basis of these experiments. It appears, however, from our experiments and that of the others, that the role of edema *per se* is not important in the pathogenesis of final tissue loss.

Finally, it should be pointed out that theoretically, the increased rate of absorption of edema fluid will simultaneously increase the absorption rate of any substances released from injured cells. These may, by their presence or concentration in general circulation, have toxic effects.

Summary and conclusions. 1. Considering that the edema formation following experimental cold injury might be a factor contributing to tissue loss, the prevention of gangrene was attempted by controlling the edema by use of the enzyme hyaluronidase. 2. Tissues edematous after cold injury showed no further swelling and a significant decline in volume after local injection of the enzyme, presumably due to an increased absorption rate of the accumulated fluid. 3. The evacuation of edema did not influence the gangrene as it was observed to develop simultaneously and equally in treated and control extremities. It seems, therefore, that tissue necrosis is not primarily related to accumulation of edema in the region of injury.

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Role of Protogen in the Oxidation of Pyruvic Acid.* (19606)

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Protogen, an essential growth factor for the ciliate *Tetrahymena geleii*(1), which is identical with the acetate replacing factor(2), and the pyruvic oxidation factor(3), has recently been highly purified(4,5), crystallized(6), and tentatively renamed lipoic acid(6). As was expected, lipoic acid was found to be capable of replacing protogen in the growth of *Tetrahymena*(7,8). The initial reaction of pyruvate oxidation may be considered as involving 2 components (as shown in reaction 1): oxi-

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dative decarboxylation, which involves a hydrogen transfer to diphosphopyridine nucleotide (DPN), and secondly, a transfer of the acetyl group to coenzyme A (Co A).

$$1.\ \text{pyruvate} + \text{Co A} + \text{DPN}_{\text{oxid.}} \rightleftharpoons \text{acetyl–Co A} + \text{DPN}_{\text{red.}} + CO_2$$

Kidder and Dewey have(9) suggested that protogen is necessary for the hydrogen transfer mechanism, whereas Gunsalus(5) holds that the factor is probably concerned with the acetylation stage of the reaction. When pure preparations of the factor recently became available[†] it was possible to initiate investigations as to the exact enzymatic role of the factor in the oxidation of pyruvate.

Methods. Pyruvic oxidase prepared by precipitation at pH 5.4(10) of a washed extract of *Tetrahymena geleii* S. was used for the study of the oxidation reaction. Assays for protogen indicated the presence of significant quantities of the growth factor in the preparation, which however could be readily removed by shaking with alumina(5). Oxidative activity was measured by spectrophotometrically following the rate of reduction of the dye, 2,6-dichlorophenol indophenol(10). The acetylation activity of the preparation was assayed by following the rate of acetyl formation by the hydroxylamine reaction(11). When the acetylation reaction was studied, the enzyme was first heated to 54°C for 10 minutes to destroy transacetylase activity(12).

Results. Removal of protogen from the pyruvic oxidase by treatment with alumina did not decrease the oxidative activity of the preparation as measured by the rate of dye reduction. In like manner, the addition of protogen to alumina treated preparations (in amounts up to 25 units/ml) had no effect. On the other hand, when the acetylation reaction was measured, protogen was found to be necessary for activity. As shown in Fig. 1, alumina treated enzyme is completely inactive in the pyruvic oxidase acetylation step. The addition of protogen to the mixture containing alumina treated enzyme restores the activity to a rate greater than obtained with untreated enzyme. It is thus apparent that the factor is necessary for the acetylation, and not the oxidative activity of the pyruvic enzyme. In the purification of the enzyme, protogen was not entirely removed, as evidenced by acetylation activity of the untreated enzyme in the absence of added protogen. The amount of the factor remaining in the preparation however, is not optimal since greater activity was obtained when excess amounts (25 units) were added to alumina treated enzyme.

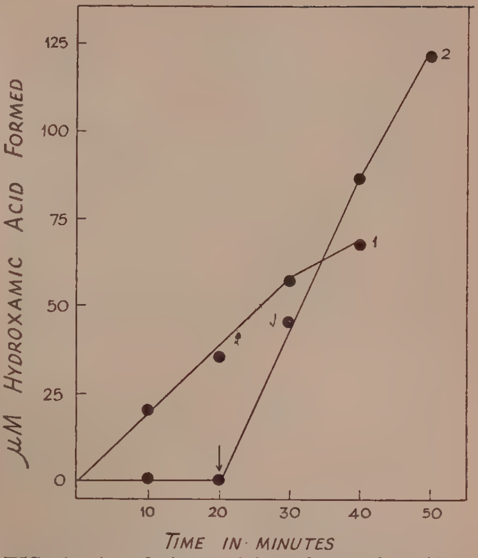

FIG. 1. Acetylation activity of pyruvic oxidase expressed as micromoles of acethydroxamic acid formed. Incubation mixture contained .2 ml of enzyme, 35 units of coenzyme A,[‡] and the following in micromoles: Sodium pyruvate, 200; cocarboxylase, .4; diphosphopyridine nucleotide, .9; $MgCl_2$, .4; $NaHCO_3$, 10; cysteine, 8.4 pH, 6.8. The final volume of the incubation mixture was 1 ml. Temp., 25°C. Curve 1, untreated enzyme. Curve 2, enzyme treated with alumina, 25 units of protogen added at time indicated by arrow.

Summary. Protogen has been found to be essential for the acetylation reaction, but not for the oxidative phase, of pyruvate oxidation, when assayed in a purified pyruvic oxidase preparation from the ciliate *Tetrahymena geleii* S.

[†] Thanks are hereby extended to Drs. E. L. R. Stokstad and L. J. Reed for making available generous quantities of protogen and lipoic acid, respectively, which were used interchangeably.

[‡] Highly purified Co A was kindly supplied by Dr. F. M. Strong.

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Effect of Nitrogen Mustard on Virus of Serum Hepatitis in Whole Blood.* (19607)

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The increasing use of blood and blood products in civilian and military medicine has brought to the fore, as one of the most urgent problems, the control of serum hepatitis (SH). The natural history of this virus disease is still obscure, but it has been definitely established that a carrier state may exist in individuals without a known history of jaundice and that such persons may harbor the virus in their blood in the absence of abnormal liver function tests(1). This situation obviously prevents the proper selection of safe donors. It is essential, therefore, to devise methods to sterilize blood and blood products. As far as plasma and sera are concerned an effective method appears to be at hand. Ultraviolet irradiation of these products has decreased the subsequent incidence of SH although the full extent of its efficacy has not as yet been critically evaluated. On the other hand, sterilization of whole blood has not been accomplished at present. In view of the relatively high incidence of the disease following transfusions the need for a satisfactory method of inactivating SH virus in whole blood is most urgent. The experience gained with nitrogen and sulfur mustards in the inactivation of various viruses seemed to offer an approach to this problem. It was first noted by Bawden and Pirie that mustards abolished the infectivity of certain plant viruses, the agents causing tobacco mosaic and bushy stunt disease, as cited by Gilman and Phillips(2). Later it was reported that sulfur mustard inactivated the viruses of rabies, Eastern equine encephalomyelitis, and hog cholera(3), and that both sulfur and nitrogen mustards rendered influenza virus non-infective(4). Herriott(5) studied the rates of inactivation of 6 animal, one plant, and 2 bacterial viruses by mustard gas and noted that those containing desoxyribose nucleic acid were more rapidly affected than the viruses containing ribose nucleic acid. Hartman *et al.*(6,7) demonstrated that the nitrogen mustard salt (methyl-bis- (beta-chloro-ethyl) amine hydrochloride) was an effective virucidal and bactericidal agent when added to plasma or whole blood in a concentration of 500 mg per liter. Plasma or blood thus treated, and subsequently neutralized with sodium thiosulfate, revealed only minimal toxicity for dogs and human beings. Studies on the components of blood indicated that the treatment induced some chemical changes which, however, in the opinion of these investigators, were not sufficiently severe to render the plasma or blood unsuitable for intravenous administration(8,10). Later it was noted that, under certain conditions, a toxic "protein-imine" (presumably formed by coupling of the dissociated chemical compound with plasma proteins) produced leucopenia in young mice when injected in large quantities(9). As a result, conditions of treatment to minimize this hazard have been recommended. The clinical use of nitrogen mustard-treated plasma has been reported by McClure *et al.*(10). No unfavorable reactions nor hematologic changes in adults or older children were observed among 400 patients given approximately 975 transfusions. It was reported that leucopenia developed in very young children transfused with the material but that these cases occurred prior to the recognition of the possibility of the formation of the toxic "protein-imine."

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* This investigation was conducted under the sponsorship of the Commission on Liver Disease, Armed Forces Epidemiological Board, Office of Surgeon General, U. S. Army.

Hampil *et al.*(11), using a technic suggested by Mangun(12) for the sterilization of whole blood[†] found that a concentration of 0.5 mg nitrogen mustard per ml of whole blood artificially contaminated with high-titered Western or Eastern equine encephalomyelitis virus did not completely inactivate either virus. However, in view of its inactivating effect on other viruses studied under these conditions of exposure, this chemical appeared to offer a possibility of success for the inactivation of SH virus. Furthermore, relatively slight chemical changes occurred in the various components of whole blood under these circumstances(11).

In the present investigation an attempt was made, therefore, to sterilize whole blood obtained in the pre-icteric stage from volunteers infected with SH virus, by treatment with nitrogen mustard prior to intravenous injection into another group of volunteers. Under the conditions of the experiment active virus remained after the treatment.

Methods and materials. Virus. The SH virus used was obtained originally from a plasma pool collected at Fort Bragg. The virus had undergone 2 passages in volunteers. Sera of individual patients of the second group, drawn between the 40th post-inoculation day and up to the dates of first development of positive liver function tests, were pooled and several such pools were combined and used, after appropriate sterility and safety tests, for infection of the blood donors needed for the present experiment. The volunteers included in the present study were healthy, young males between the ages of 21 and 33 years. Each man had a physical examination, liver function tests, complete blood count, and urine analysis prior to acceptance for the experimental study. Following inoculation, they were seen daily by a physician or a nurse. From the 40th post-inoculation day until discharged from the project, urine analyses were performed daily and liver function tests once or twice weekly. The tests have been described previously(13). *The preparation and treatment of icterogenic whole blood.* Two ml of pooled pre-icteric serum were injected intramuscularly into each of 12 volunteers. Only one of these failed to show any signs of illness, 3 developed hepatitis without jaundice, and 8 became jaundiced. The average incubation period was 81 days with a range from 68 to 88 days. On the 69th day after inoculation, *i.e.*, at the time of the first clinical signs, blood was collected from 4 of the volunteers belonging to blood group Type O, RH negative. The bottles which were to be used for collecting the infected bloods were prepared the day prior to bleeding as follows: 25 ml of ACD solution pH 4.9 were pipetted into each of a series of bleeding bottles. To half of these (labeled "Nitrogen Mustard") 0.6 ml of 2.5 M NaOH was added to give a final pH of 6.2. The alkali was calculated to be sufficient to neutralize the acidity of the nitrogen mustard hydrochloride to be added and to produce a pH of 7 to 7.2 in the presence of whole blood. The other half of the bottles not receiving the alkali were labeled "control". A vacuum was produced in all bottles and they

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[†] This technic, designed to suppress the formation of toxic substances, was identical with that employed in experiments on application of the compound to the SH blood described in this paper.

TABLE I. Results of Intravenous Injection of Nitrogen Mustard Treated Whole Blood and Non-Treated Blood into 10 Volunteers (Group B) in Each Test.

Treatment of blood	Dilution	Hepatitis with jaundice No.	Hepatitis with jaundice %	Hepatitis without jaundice No.	Hepatitis without jaundice %	Negative No.	Negative %
HN_2	Undiluted	2	20	3	30	5	50
None	”	1	10	2	20	7	70
”	1–1000	3	30	4	40	3	30
”	1–1000000	2	20	3	30	5	50

were autoclaved for 15 minutes, after which the pressure was brought down rapidly. A total of 200 ml of blood was drawn from each of the donors, 100 ml each into a “control” and “nitrogen mustard” bottle, respectively, to yield a final volume of approximately 125 ml. The bottles marked “nitrogen mustard” were kept inverted, as in bleeding, and, immediately after drawing of the blood, 6.25 ml of a 1% solution of nitrogen mustard was injected into the bottle through the stopper. This provided a final concentration of approximately 0.5 mg per ml, or 500 mg of HN_2 per liter of blood. The bloods were swirled during the addition, thoroughly agitated thereafter to insure satisfactory mixing and placed in a 37°C waterbath for 5 minutes and at room temperature for 3½ hours, thereafter.

The solution of HN_2 was prepared as follows: Weighed amounts of dry nitrogen mustard were placed into 100 ml vaccine stoppered bottles. Immediately prior to use, sterile cold pyrogen-free distilled water was injected into the bottle in sufficient amounts to make a 1% solution. Two separate bottles were used, one containing 280.7 mg of the hydrochloride salt to which 28.1 ml of water were added and another bottle containing 337.5 mg of the chemical to which 33.8 ml of water were added. The solutions were kept in a cold water bath and were used for not more than 20 minutes after preparation. Hypodermic syringes were used for making all transfers. After the 3½ hours of incubation of the bloods with HN_2, 0.6 ml of a solution of 1 M sodium thiosulfate was added to each bottle. After thorough mixing the treated and control bloods were pooled separately under aseptic conditions. The pools were then stored at 4°C for 5 days prior to transfusion in an effort to simulate conditions of storage that prevail in most blood banks.

Results of intravenous injection of HN_2-treated and untreated blood. In an evaluation of the virucidal properties of a compound the concentration of the infectious agent used obviously may influence the results. An attempt was made therefore to obtain some information as to the quantity of virus present in the blood but in experiments requiring volunteers titrations are feasible only within limits. Consequently, the 40 available volunteers were divided into 4 groups of 10 each. To the first group of 10 were given 50 ml of the nitrogen mustard treated preparation intravenously; the second group of 10 received intravenously 50 ml of the untreated preparation, both representing 40 ml of whole blood. Groups 3 and 4 received 40 ml of 10^{-3} and 10^{-6} dilutions of untreated preparation, respectively, or 0.04 and 0.00004 ml of whole blood.

The results of this experiment are shown in Table I. It is seen that the nitrogen mustard as used was ineffectual in preventing serum hepatitis. There were 2 cases of hepatitis with jaundice and 3 without jaundice resulting from injection of the nitrogen mustard-treated blood as compared to one case with jaundice and 2 without jaundice in the control group. In the 2 groups given control blood diluted to 10^{-3} and 10^{-6} there were 3 and 2 cases of hepatitis with jaundice and 4 and 3 of hepatitis without jaundice, respectively. The average incubation period was 78.6 days with a range of 68 to 84 days. There were no toxic effects noted in the 10 men who received the nitrogen mustard-treated blood.

Discussion. On the basis of the inoculation of 50 ml of untreated blood diluted to 10^{-6} and a correction for the dilution factor introduced by the ACD solution 1 ml of the pooled blood contained at least 25,000 infectious doses of SH virus and possibly more. There is no doubt that nitrogen mustard at a concentration of 500 mg per liter failed to render the blood safe for transfusion.

In evaluating this result several possible factors must be considered. 1. It may be that nitrogen mustard failed to combine with the protein of the SH virus and thus failed completely to inactivate. This is not compatible with the suggestive results obtained with icterogenic plasma treated with nitrogen mustard(7). 2. It is not known whether the virus may not be harbored within erythrocytes or white blood cells. If it is located inside cellular components of the blood it is possible that the concentration of nitrogen mustard within the respective cells was too low and thus the virus particles were not inactivated. 3. It is conceivable that the nitrogen mustard may have inactivated a large proportion of the virus particles but there remained enough in the active state to induce infection in the volunteers. As a matter of fact only 1 in 10^6 particles may have escaped inactivation in the experiment reported. This interpretation could have been verified only by titrating also the HN_2 treated blood. In any case, such highly infectious bloods presumably may be drawn by chance from blood donors in the late incubation period or possibly even from carriers of SH virus in the routine collection for blood banks. The donors of the infective blood in this study were bled 3-9 days prior to the onset of jaundice, in the assumption that the preicteric stage may represent the time at which maximal titers of virus are obtained in the blood. However, it is not known whether this might not occur earlier in the incubation period. It is likely that this experiment constituted a particularly severe test for the inactivating capacity of HN_2 and that possibly under routine conditions, where asymptomatic carriers and not incubatory carriers may be the prime source of infection, the HN_2 treatment may have given more encouraging results. However, it is obvious that the ideal sterilizing agent of whole blood should be effective against maximal possible concentrations of SH virus.

The result obtained with the technic of HN_2 treatment used was similar to those noted with some other viruses, notably the agents of Western and Eastern equine encephalomyelitis. These were not completely inactivated in artificially contaminated whole blood by HN_2, whereas other viruses became non-infective. Thus, it would seem that SH virus belongs to the more resistant viruses, which would be compatible with other information with regard to its stability. Higher concentrations of HN_2 and longer exposure of SH virus to this chemical might prove to be more effective but in that case the danger of toxic byproducts of the reaction and changes in the blood constituents may prevent the safe use of the blood for transfusions.

Summary. 1. Nitrogen mustard in a concentration of 500 mg per l failed to inactivate the Fort Bragg strain of serum hepatitis virus in whole blood. 2. Possible reasons for this failure are discussed. 3. The virus titer of the whole blood used was found to be at least 25000 infectious units/ml.

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Effect of Hyaluronidase on Reaction of Rabbits to Intradermal Inoculations of Vaccinia Virus.* (19608)

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When rabbits were injected intradermally with spreading factor from testicular extracts (hyaluronidase) together with concentrated suspensions of bacteria, the resulting lesions were more widespread than those produced by the inoculation of equally concentrated suspensions of bacteria in saline. Later observations, however, indicated that inoculations of testicular extract with dilute bacterial suspensions either induced lesions which were less widespread than those induced by saline suspensions or failed to induce a lesion (1,2). These data led to the conclusion that in the case of bacteria there could be determined a *critical concentration,* the minimal effective concentration per unit area of infection, below which no lesions would occur, and above which the intensity of the lesions would be roughly proportional to the concentration. However, the lesions induced by highly virulent bacteria such as some pneumococcus strains failed to be suppressed by spreading, even if injected in amounts nearing the minimal infective dose (presumably a single bacterial cell), but if antiserum was injected intravenously then pronounced suppression of the lesions by spreading occurred. When several viruses were examined, it was observed that no suppression took place as a result of the dispersion of the virus either in normal rabbits, or in rabbits injected with immune serum. It was then concluded that there is not a *critical concentration* for the viruses studied as there is for bacteria, or that the *critical concentration* of virus per unit area of tissue is an infective dose of virus.

The data recorded in the present paper indicate that when the resistance of rabbits is increased by virtue of a previous infection with vaccinia virus, subsequent intradermal inoculation of the virus together with hyaluronidase or testicular extract results in partial suppression of the lesions. This finding is supported by a decrease in the endpoint of intradermal infectivity for the rabbit of the lesions of the hyaluronidase series as contrasted with the saline series and by an increase in the ratio of the diameter of the lesions of the saline series of virus dilutions to the diameter of the lesions of the hyaluronidase series in rabbits with a partial, active immunity as contrasted with the ratio in non-immunized and passively immunized rabbits.

Materials and methods. Ten-fold serial dilutions of the commercial "calf lymph" strain of vaccinia virus were made in saline, and each dilution divided into 2 portions. To one portion was added an equal volume of saline buffered at pH 7.2 with M/15 phosphate buffer, and to the other an equal volume of 2% purified hyaluronidase (Wyeth) or a 10% extract of rat testes also buffered at pH 7.2. These 2 sets of virus dilutions were then inoculated intradermally in a volume of 0.1 ml in non-immunized rabbits, in rabbits passively immunized with hyperimmune anti-vaccinia serum, and in rabbits which had been infected intradermally with vaccinia virus 2 to 16 months previously. The saline series of virus dilutions were injected on the right side, and the hyaluronidase or testicular extract series on the left side of the rabbit. The hyaluronidase usually contained between 1000 and 1500 turbidity reducing units per mg. The extract of rat testes was prepared and used on the same day. The passively immunized group of rabbits received, depending on the lot of serum and the weight of the rabbit, from 4 to 20 ml of immune serum intraperitoneally from 1 to 6 days prior to the virus. Each lot of serum represented a pool obtained from 3 or more rabbits which were convalescing from a second infection. The animals were bled from 2 weeks to one month following the second infection. The titer of

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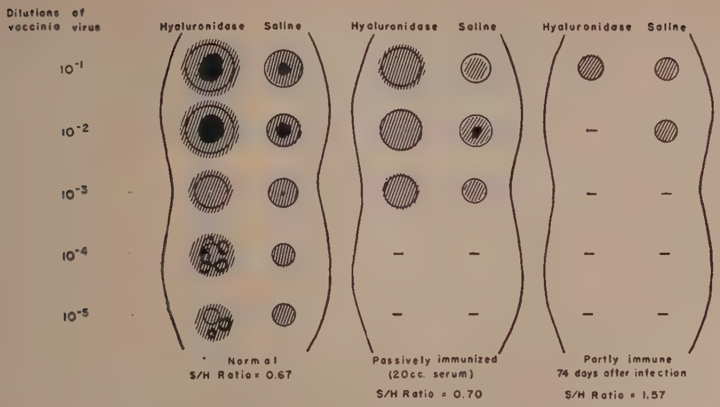

FIG. 1. Vaccinial lesions in rabbits recorded after five days.

hemagglutination-inhibiting antibodies varied from 320 to 2560. The dose of serum was adjusted so that each rabbit received approximately the same concentration of anti-vaccinia antibodies. The resulting lesions in all groups of rabbits were calibrated at frequent intervals following inoculation. The diameter of the lesion was ascertained by adding the smallest and largest diameters and dividing by two. Only those lesions which were at least 5 mm in diameter were considered to be produced specifically by vaccinia virus. Due to the marked variability in resistance of rabbits to intradermal inoculations of vaccinia virus, the experimental results are expressed largely in terms of the ratio of the diameter of the lesions of the saline series to the diameter of the lesions of the hyaluronidase series (S/H ratio). With this method it was found that the ratios within a particular group of inoculated rabbits was remarkably constant despite the fact that there might be a 2 or 3 log difference in the endpoint of intradermal infectivity for the rabbits included in the group.

Results. Despite the use of different lots of hyaluronidase, a number of testicular extracts, several samples of hyperimmune serum administered from 1 to 6 days prior to the virus, and rabbits which had been infected with vaccinia virus 2 to 16 months previously, the data obtained in each of the 8 experiments proved to be remarkably consistent. The results of a typical experiment are illustrated in Fig. 1. In the normal and the passively immunized rabbits hyaluronidase enhanced the vaccinial lesions induced by every dilution of the virus. In the previously infected rabbit hyaluronidase actually suppressed the development of the lesion when the 10^{-2} dilution of vaccinia virus was employed, and did not enhance or frankly suppress the lesion induced by the 10^{-1} dilution of virus. It should be noted that the diameter of the lesions in the saline series of virus dilutions was essentially 70% of that found in the hyaluronidase series both in the normal and in the passively immunized rabbits despite the fact that there was a 2 log decrease in the virus titer in the passively immunized rabbit. Thus, although the resistance of the rabbit was appreciably increased by the administration of the hyperimmune serum, the effect of hyaluronidase on the reaction of the rabbit to intradermal inoculation of vaccinia virus was the same as that observed in the non-immunized rabbit. On the other hand, when the resistance of the rabbit was increased by a previous infection with the virus, the diameter of the lesions in the saline series of virus dilutions was 157% of that encountered in the hyaluronidase series indicating a suppressive effect of hyaluronidase on the development of the lesions.

The data obtained in the 8 experiments are summarized in Fig. 2. When the reactions of the rabbits are expressed in terms of the ratio of the diameter of the lesions of the saline series to the diameter of the lesions of the

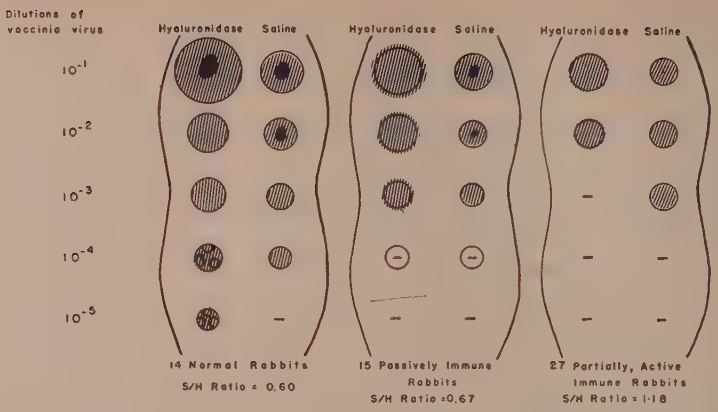

FIG. 2. Summary of vaccinial lesions in non-immune and partially immune rabbits.

hyaluronidase or testicular extract series (S/H ratio), the ratios are 0.60, 0.67, and 1.18 for the non-immunized, the passively immunized, and the partially, active immune groups of rabbits, respectively. In addition to the enhancing effect of hyaluronidase on the development of vaccinial lesions in the non-immunized and passively immunized rabbits and its suppressive effect in the previously infected rabbits, it was observed that hyaluronidase decreased the extent of necrosis which is depicted in Fig. 2 as the solid black area in the center of the lesion. The ability of hyaluronidase to give rise to isolated lesions in the non-immunized rabbits (10^{-4} and 10^{-5} dilutions of virus) is indicated by the smaller circles within the larger area of the lesion. As indicated by the open circle the lesions in the passively immunized rabbits induced by the 10^{-4} dilution of virus had a tendency after the 5th day to fade rapidly.

When the reactions of the rabbits to intradermal inoculations of vaccinia virus are tabulated in terms of days after infection, the S/H ratios remain relatively constant (Table I and Table II). The possibility cannot be excluded that the 2 passively immunized rabbits listed in Table I which had an S/H ratio of 0.89 also had a contact infection with vaccinia virus within the week prior to inoculation. Otherwise, the S/H ratios of the non-immunized and the passively immunized groups of rabbits are similar. In the previously infected rabbits in Table II the S/H ratios are consistently higher than the controls. The markedly higher S/H ratios found on the 8th and 9th days are a reflection of the fact that the lesions in the hyaluronidase series tend to fade much more rapidly than the lesions in the saline series of virus dilutions. Although the lesions in the passively immunized rabbits induced by the higher dilution of virus also fade, there is no marked difference between the rate of disappearance of the lesions in the hyaluronidase series as compared with the saline series of virus dilutions.

That the lesions in the partially, active immune rabbits are the result of an actual infection rather than an allergic manifestation due to the previous infection is indicated by the following facts. The evolution of the lesion, especially in the saline series of virus dilutions, follows the same pattern as that observed in the non-immunized and the passively immunized rabbits. Virus has been isolated from the lesions in the partially, active immune rabbits in approximaely the same concentration as in comparable lesions in the passively immunized rabbits. Finally, heat inactivated or partially heat inactivated vaccinia virus has failed to induce lesions in the partially, active immune rabbits.

The regularity with which vaccinial lesions were induced in rabbits which had been infected intradermally with vaccinia virus 2 to 3 months previously was an interesting finding. This was somewhat surprising in view of the

TABLE I. Reaction of Passively Immunized Rabbits to Intradermal Inoculations of Vaccinia Virus.

Time, days	No. rabbits Immunized	No. rabbits Controls	S/H ratio* Immunized	S/H ratio* Controls
2	3	2	.56	.70
3	11	6	.68	.64
4	2†	2	.89	.57
5	14	8	.64	.57
6	3	2	.53	.48
7	9	4	.62	.54
8	3	2	.66	.43
9	3	2	.57	.43

* The ratio of diameter of lesions of the saline series of virus dilutions over the diameter of the lesions of the hyaluronidase or testicular extract series.

† These 2 rabbits may have had a contact infection within the week prior to inoculation since they were mistakenly placed in a room with rabbits infected with vaccinia virus.

TABLE II. Reaction of Previously Infected Rabbits to Intradermal Inoculations of Vaccinia Virus.

Time, days	No. rabbits Immunized	No. rabbits Controls	S/H ratio* Immunized	S/H ratio* Controls
2	8	3	1.20	.63
3	19	7	1.07	.63
4	5	2	1.20	.57
5	15	6	1.18	.53
6	8	3	1.54	.50
7	11	4	1.06	.64
8	5	2	3.69	.43
9	10	4	2.27	.51

* The ratio of diameter of lesions of the saline series of virus dilutions over the diameter of the lesions of the hyaluronidase or testicular extract series.

high titers of neutralizing antibodies present in the serum of the rabbits, and especially in view of the continued presence of the virus in the testes of these rabbits. In 2 experiments testes were removed from rabbits which had been infected intradermally with vaccinia virus 53 to 86 days previously. In 9 of 10 cases vaccinia virus was isolated from the testes by inoculation of the chorioallantois of the chick embryo. Three isolations of virus were made on the first egg passage and 6 on the second egg passage. Virus was not isolated from 4 control testes after 3 egg passages. These rabbits developed typical lesions when inoculated with vaccinia virus on the day following the removal of the testes. Pearce(3) has isolated vaccinia virus from rabbits as long as 17 weeks after infection.

Comment. The suppressive effect of hyaluronidase on the intradermal inoculation of vaccinia virus in partially, active immune rabbits may be due to a spreading of the virus over a larger area and thus effectively increasing the concentration of neutralizing antibodies per unit of virus. If this explanation is correct, then it appears necessary to assume that the distribution of antibody is different in the passively immunized rabbit than in the partially, active immune rabbit for it was observed that hyaluronidase enhances vaccinial lesions in passively immunized rabbits. The possibility that other defensive agencies are acting in concert with the antibody has to be considered. The recent demonstration that hyaluronic acid inactivates vaccinia virus(4) suggests the intriguing possibility that a direct toxic effect on the virus by altered hyaluronic acid may play a role in the observed phenomenon. Whatever the mechanism involved in the inactivation of the virus, the important point shown by the present investigation is that there is a *critical concentration* for at least one virus, as there is for bacteria. However, in contrast to what is observed with the latter, the reality of this *critical concentration* can only be shown in hosts endowed with an active, partial immunity.

Summary. 1. Hyaluronidase or testicular extract enhances the lesions produced in non-immunized and passively immunized rabbits following intradermal inoculation of vaccinia virus. 2. On the contrary, in rabbits with a partial active immunity the lesions induced by the virus are partially suppressed by the spreading effect of hyaluronidase. 3. When the reactions of the rabbits are expressed in terms of the ratio of the diameter of the lesions of the saline series of virus dilutions to the diameter of the hyaluronidase or testicular extract series, the ratios are 0.60, 0.67, and 1.18 for the non-immunized, the passively immunized, and the partially, active immune groups of rabbits, respectively.

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Influence of Body Fat on Duration of Thiopental Anesthesia. (19609)

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Brodie *et al.*(1) have shown that fat tissue has a high affinity for thiopental. Theoretically the amount of fat in the body should therefore influence the course of thiopental anesthesia. DeBoer(2) found that semi-starvation prolonged the sleeping time of dogs under thiopental anesthesia. This study was undertaken to see whether an effect of body fat on thiopental anesthesia is demonstrable and of sufficient magnitude to be of practical importance.

Experimental procedure. A fat group (F) and a lean group (L) of albino rats were prepared over a period of 30 days from an originally homogeneous group of rats. The fat group received *ad lib.* a mixture of Purina Dog Chow, and lard (30%) supplemented with choline chloride (0.1%) and thiamine (.017%). The lean group was individually caged and fed daily, on an individual basis, the amount of Purina Dog Chow which would induce a progressive small weight loss. The average starting and final weights of Group F were, respectively, 175 g and 199.3 g. Average starting and final weights for Group L were, respectively, 188.6 g and 169.0 g. Both groups appeared active and healthy at all times. Thiopental (1.4% solution in isotonic saline) was injected into the tail vein; pentobarbital (2% solution in isotonic saline) was administered intraperitoneally. No rat received more than one dose of an anesthetic in a 48-hour period. The time from loss of righting reflex until regaining of righting reflex was taken as the duration of anesthesia. The amount of body fat was estimated on the shaved, eviscerated animal by the specific gravity method developed by Rathbun and Pace(3).

Results and conclusions. The "fat" group of animals were found to contain 5 g % more fat than the "lean" group (Table I). This presumably represents at least a doubling of the adipose fat, since much of the fat of the lean group must be represented by phospholipids, cholesterol, etc.

The duration of thiopental anesthesia was more than doubled in the lean rats compared to the fat rats. It seems fair to ascribe this to the observed difference in body fat since the duration of pentobarbital anesthesia was identical in both groups. The difference in thiopental anesthesia can scarcely be attributed to possible difference in liver function in the 2 groups since Shideman and Gould(4) found that the rat destroys thiopental at a rate of only 10% per hour.

The relative dosage of thiopental required by the fat and lean rats for a given period of anesthesia was estimated by dosage response curves (Fig. 1). It is seen that the dose for a given period of anesthesia was about 40% greater for the fat animals. This would be explained if the 5 g % extra fat of these animals took up 8 times as much thiopental as the rest of the body. Brodie *et al.*(1) found that the adipose fat of the dog will take up 6 to 10 times as much thiopental as other parts of the body.

On the basis of the present data it would seem worth exploring the possibility that thiopental anesthesia in man would be more predictable if the dosage were adjusted with ref-

TABLE I. Effect of Body Fat on Anesthesia with Thiopental and Pentobarbital.

	F Fat group	L Lean group	L–F	Probability
No. of animals	17	18		
Body fat, %	13.5 ± .8*	8.5 ± .5*	— 5 ± 1 *	<.001
Duration of anesthesia (min)				
Thiopental, 30 mg/kg	23.5 ± 1.8	52.6 ± 6.3	+29.1 ± 6.5	<.001
Pentobarbital, 25 mg/kg	26.7 ± 4.2	26.7 ± 4.3	0 ± 6	1

* ± stand. error.

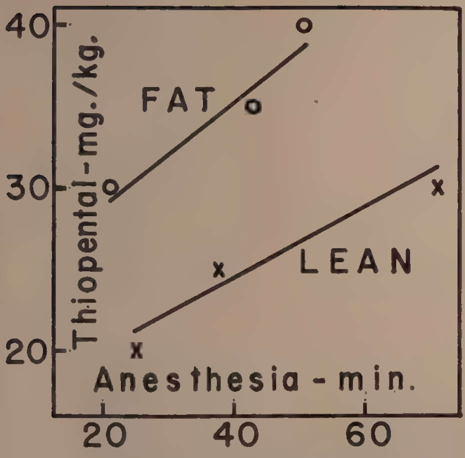

FIG. 1. Relationships between duration of anesthesia and thiopental dosage.

erence to the body fat.

Summary. A 5 g % decrease in body fat of the rat resulted in a 100% increase in length of thiopental anesthesia at a fixed thiopental dosage, or a 40% decrease in the amount of thiopental required for a given duration of anesthesia. In contrast the duration of pentobarbital anesthesia was unaffected by the amount of body fat.

We wish to express our appreciation for the suggestions and criticism received from Dr. Oliver H. Lowry and Dr. Morris Friedkin throughout the course of this investigation.

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Casein and Factor 3 in Dietary Necrotic Liver Degeneration; Concentration of Factor 3 from Casein. (19610)

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It is a general experience that standard vit. E-free test diets which contain 10% or more of so-called "vit. free," *i.e.*, alcohol-extracted casein cannot be used for the production of dietary necrotic liver degeneration in rats. The deficiency does develop, however, when the animals are raised on rations containing only 4 to 6% of the "vit. free" casein(1,2). A purified, alkali-treated Hammarsten* casein ("casein VI"), on the other hand, had been used over a period of years at levels of 15 and even 30% for the production of dietary necrotic liver degeneration in experiments which led in 1944 to the detection of the protective activity of vit. E(3,4). It was stated at that time that "the most important condition for the development of the liver damage is to be seen in the nature of the casein used," and that "the occurrence of protective activity in completely dissimilar materials demonstrates that several different substances must exist which have an effect. . . ." (3). Evidence accumulated thereafter for a protective substance which was removed during the preparation of casein VI and which was different from cystine or from vit. E remained unpublished.† Recently, however, we reported the presence of such a factor in certain yeasts(6). The active principle, designated factor 3, prevents the development of dietary necrotic liver degeneration and is found in a wide variety of natural sources.‡

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* "Hammarsten" casein (O. Hammarsten 1877, l. c. 7.) is prepared from freshly obtained casein by repeated acid precipitation from dilute alkaline solution and final drying by ethanol and ether.

† See discussion in: Liver Injury, Transactions of the Eighth Conference, Josiah Macy, Jr., Foundation, New York, 1949, p49.

‡ To be published separately.

TABLE I. Protective Effect of Casein and Casein Preparations.

Exp.	Supplement	No. of animals	Dead due to liver necrosis	Avg reciprocal survival time (×100)	Activity, units/g of supplement
A*	0	8	8	2.00 ± .19‖	—
	Crude casein (3%)	9	2	.32 ± .25	9
	"Vit. free" casein a (3%)‡	10	2	.19 ± .12	9.6
B*	0	9	9	2.19 ± .19	—
	"Vit. free" casein b (3%)‡	10	3	.72 ± .41	7.2
	Amino acid mixture (3.6%, ≅ 3% casein)§	10	10	2.03 ± .31	0
C*	0	9	9	2.10 ± .09	—
	Alcohol extr. obtained during casein b production (≅ 5%)	10	10	2.22 ± .13	0
D*	0	9	9	2.74 ± .22	—
	"Vit. free" casein b (3%)‡	10	7	1.19 ± .31	6
	Hammarsten casein prepared from casein b (3%)	10	9	1.98 ± .31	3
E†	0	9	9	2.51 ± .24	—
	Bacto Casitone (2%)‡	10	6	1.1 ± .32	9

* Prophylactic method. † Repletion method (supplementation started 24th day). ‡ Commercial sample. § Composed according to figures given by R. J. Block and D. Bolling, The Amino Acid Composition of Proteins and Foods, C. C. Thomas, Springfield, Ill., Ed. 1945, p. 303. ‖ Mean ± stand. error.

TABLE II. Purification of Active Substance from Bacto Casitone. (Tests by repletion method.)

	Dry wt, mg/g of starting material	Activity		Approximate conc.
		u/g of starting material	u/g of dry wt	
Bacto Casitone	1000	10.5	10.5	1 : 1
Charcoal filtrate*				
Charcoal eluate	356	10.5	29.6	2.8 : 1
Alcohol precipitate from charcoal eluate	261	3.7	14.4	1.4 : 1
Alcohol filtrate	68.4	3.8	56	5.3 : 1
Precipitate from water sol. of alcohol filtrate	12.2	4	320	30 : 1
Water filtrate after separation of precipitate	88†	1.6	27	2.6 : 1

* Not tested. † After neutralization.

In this paper evidence is presented for the occurrence of factor 3 in crude and purified samples of casein and of enzymatic casein digests. The 30-fold concentration of the active substance from an enzymatic casein hydrolysate is described.

Experimental. a) *Animal experiments.* Details concerning the animal experiments were reported previously(5,6). Male Sprague-Dawley rats of the National Institutes of Health colony were used. The animals were weaned at 21 to 24 days of age, at which time their average body weight was 41 g. Litter-matched groups of 10 rats of equal average weight were formed. In each experiment one group was fed the basal ration as a control. The basal diet consisted of 30% torula yeast, 59% sucrose, 5% salts, 5% vit. free lard and a vitamin mixture(5). From this ration the experimental diets were obtained by the addition of supplements at the expense of sucrose. Tests for protective activity were performed either by a prophylactic method (Exp. A-D, Table I), *i.e.*, supplementation was started immediately after weaning, or by a repletion method (Table II), in which case supplementation started after a period of 23 days on the basal diet(6). To express the speed of development of the deficiency, the average of the reciprocal survival time was employed and compared, as described before(3,5). With increasing dietary levels of supplements containing factor 3, the reciprocal survival time decreased. A straight line relationship was observed over a wide range of dosage.[‡] Protective effects were evaluated in units (u), one u being defined as the amount of factor 3 required per day per animal to effect a 50% reduction of the average reciprocal survival time as compared to the unsupplemented littermate control group. In other words, one u of factor 3 per day per animal doubled the (virtual) average survival time of the rats. It was found, for example, that 1 u was contained in about 0.1 g of Bacto Casitone.[§] It is recognized that the test method used does not represent a perfect biological assay procedure. It permits, however, an estimation of the approximate factor 3 activity of natural materials and preparations therefrom. Repeated tests yielded results consistent within a range of ± 15%.

b) *Concentration procedure.* One thousand g of Bacto Casitone were dissolved in 6 liters of water, the pH was adjusted to 8.7 by the addition of 6 N NaOH, the temperature of the solution was brought to 25°C and 1000 g of charcoal (Darco KB) were added with gentle stirring. The pH dropped below 8 and was brought back to 8.7 by 6 N NaOH before filtration (Buchner funnel). The filtrate was again adjusted to pH 8.7, diluted with 2 liters of water and treated a second time with charcoal as described above. The combined charcoal adsorbates were washed with 2 liters of water with stirring, the pH was kept at 8.5 by the addition of 6 N NaOH. After filtration the charcoal was stirred with 5 liters of ethanol at 50-55°C for 30 minutes. The alcohol elution was repeated. The combined ethanol eluates were evaporated under nitrogen to a sirup which was made up to 1 liter with water ("charcoal eluate"). Nine liters of ethanol were added slowly to the "charcoal eluate" with constant stirring. The precipitate was redissolved in 1 liter of water and precipitated again with 9 liters of ethanol. The mother liquors of the first and second precipitations were combined, cleared from turbidity by filtration and concentrated to a sirup which was taken up in 200 cc of hot water ("alcohol filtrate").

The water solution of the alcohol filtrate was kept at +4°C for 24-48 hours. The heavy, creamy precipitate which formed was separated by filtration through a Buchner funnel ("precipitate from water"). This fraction was about 30 times as active as the starting material, as shown below.

Results. Factor 3 activity of casein and preparations. Addition of 3% crude casein and also of various commercial samples of alcohol-extracted "vitamin free" casein at a 3% level to the torula ration greatly inhibited the development of necrotic liver degeneration (Table I, Exp. A, B, and D). The 2 samples of "vit. free" casein of different origin showed an activity similar to that of crude casein. Substitution of an equivalent amount of amino acids, imitating the composition of casein, was without effect (Exp. B).

The alcohol extract obtained during the commercial production of "vit. free" casein was tested and found to be inactive (Exp. C). This shows that factor 3 is not removed to any extent from casein by continuous alcohol extraction.

When "vit. free" casein was purified by the procedure originated by Hammarsten(7), *i.e.*, by solution in alkali and reprecipitation with acid, about half of the "factor 3" activity was eliminated (Exp. D).

Commercial enzymatic casein digests, such as are widely used as ingredients in bacteriological media, showed an activity similar to that of casein, for example, Exp. E, Table I.

It is obvious from these findings that crude and also commercial alcohol-extracted "vit. free" casein contain a component actively protecting against the deficiency. That the effect must be attributed to a factor other than vit. E or cystine, the 2 established protective substances, was derived from the following considerations: "Vit. free" casein does not contain vit. E since such casein is a standard component of rations used for the production of vit. E deficiencies. The quantity of cystine (and methionine), on the other hand, which is supplied by the addition of 3% of casein to the ration is too small to be of consequence. It was found that levels of 0.2% or more of cystine in the diet are required to influence the course of necrotic liver degeneration under our experimental conditions(5).

More direct proof for the existence of factor 3 in casein and for its distinctness from vit. E and from cystine is provided by the fractionation of the active material.

Concentration of factor 3. In enzymatic casein hydrolysates, factor 3 appears to be, at least in part, present in an easily accessible, low molecular form. Concentration of factor 3 was, therefore, initiated from an enzymatic casein digest as starting material. The animal test was used as assay procedure. The first 3 steps of concentration, described in detail above, have been used repeatedly with only slight variations. Results of a typical run are given in Table II. In order to avoid losing activity in the first step of the concentration process, the material was absorbed on relatively large amounts of charcoal from which the active substance could be removed by repeated treatment with hot ethanol. A loss of activity was not apparent during this process; the charcoal eluate contained about one-third of the original dry weight. Precipitation of inactive impurities, mostly peptides, by ethanol was employed as the next step. Half of the activity remained in solution, the other half was lost in the precipitate, which possibly contains a bound form of factor 3. From the "alcohol filtrate" further purification of factor 3 was provided by the separation of a portion less soluble in water. This product, comprising some organic and inorganic cations, amino acids, and other organic acids shows a sulfur content of less than 0.5%. It is 30 times as active as the starting material and is being used for the further purification of factor 3.

[§] Difco Laboratories, Detroit, Mich.

Discussion. The results show that casein contains factor 3. So far no essential difference between the active principle concentrated either from yeast or from casein has been encountered(8). The question, however, whether the active substance from these 2 sources is completely identical remains to be clarified. Both crude and "vit. free" casein compare favorably with the best known sources for factor 3, for example, with yeast K(5). It is apparent that alcohol extraction, the method used for the production of "vit. free" casein, does not remove factor 3. The customary vit. E free diets do not lead to necrotic liver degeneration, evidently because protection is afforded by the amount of factor 3 present in the "vit. free" casein.

Acid precipitation of casein from alkaline solution ("Hammarsten" procedure(7)) partly removed factor 3. This might explain why "casein VI" diets could be used for the induction of the deficiency in the older experiments. At that time "casein VI" had been prepared from a repeatedly reprecipitated "Hammarsten" casein by additional treatment with alkali in the heat and subsequent acid precipitation(3). For the preparation of casein VI, the purity of the starting material appears to be a decisive factor. Several attempts to produce casein VI from alcohol-extracted "vit. free" casein were unsuccessful(8-10).

A potential discrepancy might be seen between the protective effect elicited by the addition of 3% casein to the torula diet and the older findings that diets containing 4, 6, or even 10% casein produced the deficiency. This might be explainable on the basis of differences in the factor 3 contents of casein samples employed at various times. Whether other reasons are involved, however, has to be clarified by further experimentation. It is obvious, however, that the protective effect of casein on dietary necrotic liver degeneration is not due simply to the improvement of the protein supply; it is of a more specific nature.

Summary. 1. Samples of crude and "vit. free," alcohol-extracted casein, and also enzymatic casein digests, were found to contain considerable amounts of factor 3, which protects against dietary necrotic liver degeneration. This explains why the deficiency does not develop on customary vit. E free casein diets. 2. A method is described for the concentration of factor 3 from an enzymatic casein hydrolysate, affording a 30-fold purification in 3 steps, with an over-all recovery of about 38%. This concentrate is being used for the further purification of factor 3.

Acknowledgment is made to Samuel M. Takahashi and Joseph D. Mullineaux for efficient technical assistance.

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Adenosine Triphosphatase Activity of the Vascular System.* (19611)

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The contractile mechanism which specializes skeletal muscle for quick movement is presumably the same as that which enables smooth muscle to maintain its tonus state. It has been shown that the energy-rich phosphate bonds of adenosine triphosphate are the sole immediate source of energy for skeletal muscle contraction. In addition, evidence points to the fact that ATP plays a central role in the maintenance of smooth muscle tone and in the initiation of its contraction. Dubois and Potter(1) devised a method to determine the amount of adenosine triphosphatase (ATPase), the enzyme which is responsible for ATP hydrolysis, in various tissues. Krantz, Carr and Bryant(2) showed that the acutely-acting vasodilators of the nitrite-nitrate series inactivated ATPases present in the aorta of rabbits. The interference with enzyme activity occurred with therapeutic levels of the vasodilators. Thus it appears possible that these vasodilators may exert their action through the ATP-ATPase mechanism as the target enzyme system in arterial tissue.

Owing to this observation we considered it of interest to study the ATPase activity of various segments of the vascular bed. Furthermore, the normal variations within a single species of animal appeared important. In addition, the variation of the vascular ATPase activity of different species was determined, and the effect of age upon the enzyme concentration in the vascular wall was estimated.

Method. The principal studies were carried out on male mongrel dogs approximately in middle life. The animals were maintained on a balanced laboratory ration for a period of several months until a good nutritional state was established. The animals were anesthetized with an intravenous dose of pentobarbital sodium, and the vessels removed immediately after anesthesia ensued. It was previously determined that this barbiturate did not affect the ATPase activity of the vessels. All determinations were carried out within one-half hour after tissue removal. It was determined that during this period the ATPase activity did not undergo diminution. This same procedure with suitable modifications, to adapt it to the various species, was employed in all animals studied, omitting barbiturate anesthesia. Rats were used for the studies concerning the influence of age on the enzyme owing to the ease of obtaining animals of definite age. The ATPase activity of the tissues was determined by the method of Bell, Carr and Krantz(3). This method employs the tissue incubation of DuBois and Potter, the precipitation of the phosphate as magnesium ammonium phosphate recommended by Griswold(4), and the use of ammonium sulfate as suggested by Lowry and Lopez(5). The recovery of phosphate from tissue by the method is approximately 90%. The reproducibility of the method on a single specimen of tissue is shown by a standard deviation of 0.18 unit and a coefficient of variability of 3%. The values are expressed in ATPase units as adopted by DuBois and Potter, *i.e.*, the number of gam-

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* The expense of this investigation was defrayed in part by a grant from the National Institutes of Health.

TABLE I. ATPase Activity of Vascular System of the Dog.

	No. of animals	High	Low	Mean	S.E.
Arterial tissue					
Aorta	21	16.5	7	11.6	±.7
Carotid	21	8.5	1.5	5.1	±.5
Coronary	32	8.1	1	4.6	±.4
Renal	19	7.7	2	4.8	±.3
Femoral	20	7.3	1	3.3	±.4
Venous tissue					
Jugular	19	No activity			
Renal	1				
Brachial	1				
Pulmonary	1				
Femoral	1				
Vena cava	1				

TABLE II. Aortic ATPase of Young and Old Male Rats.

Group No.	Wt, g	No. of rats	ATP units
	Young rats		
1	50–60	3	26
2	"	3	26
3	45–55	4	49
4	"	4	32
5	"	4	24
	Young adult rats		
1	150–180	1	34
2	"	2	36
3	150–200	3	29
	Old rats		
1	450–500	1	41
2	"	1	34
3	"	1	26
4	430–500	2	28
5	"	2	31

TABLE III. Aortic ATPase of Various Species.

	Animal No.	ATP units
Chelonia mydes—turtle	1	0
	2	0
Rana virescens—frog	1	7.8
	2	8.5
	3	4
Gallus domesticus—chicken	1	1.5
	2	>1
	3	>1
Epemys norvegicus—wild rat	1	22
	2	22
	3	21
Cavia cobaya—guinea pig	1	7
	2	10.5
	3	8.7
Lepus cuniculus—rabbit	1	5
	2	5.8
	3	6.3

ma of inorganic phosphorus liberated by 1 mg of tissue in 15 minutes at 37°. The value expressed as ATPase activity may be due to one or more enzymes present in the tissue as pointed out by Swanson(6). For example, liver has been shown to contain several kinds of enzymes measured as ATPase. In our incubation system we have maintained a pH value of 7.4 and Ca^{++} was used as the activator. The enzyme or enzymes acting under these conditions have been considered a measure of the ATPase tissue activity.

Results. (See Tables 1, 2, 3)

Discussion. An examination of Table I reveals that the distribution of ATPase varies greatly in the arterial system. From our studies in the dog, the venous system is apparently devoid of the enzyme. Kováts(7) found the actomyosin content of different parts of the heart proportional to their mechanical activity. Csapó(8) observed that the uterus was relatively poor in actomyosin until pregnancy occurred. During pregnancy it was observed that the actomyosin activity increased and its distribution bore a direct relation to the functional activity of the organ during gestation and at term. Similarly, the vascular system of the dog enjoys a broad variation in ATPase activity. Nevertheless the data clearly show that the enzyme is present in greatest quantity in the aorta. The coronary, carotid and renal arteries appear to share about equally in the enzyme distribution. It has been shown by Baló, Banga and Josepovits(9) that the human aorta contains from 20 to 31% of water-soluble protein, and it is this portion of aortic tissue which contains ATPase.

The rat's aorta (Table II) contains a very high concentration of ATPase. It appears from our studies that this value is not affected by age or captivity in this species.

Our principal concern in these studies is the relationship of the concentration of ATPase in arteries to the phenomena of vasoconstriction and the action of vasodilators. Indeed it appears that vasodilators which inhibit ATPase activity might have ATP-ATPase as their target enzyme system and through this inhibition elicit their response. One may postulate that the inactivation of ATPase prevents ATP from hydrolyzing and thus deprives the arterial tissue of the energy which is released from the high energy phosphate bond. If through the decomposition of ATP by its enzyme ATPase, tonus and constriction of the artery are maintained, it is possible that a drug which acutely relaxes the artery may do so by interfering with the ATPase activity of the tissue. Studies aimed at clarifying this postulate are in progress.

Summary. The ATPase activity of the vascular system of the dog has been studied. It has been shown that the enzyme content of the different arteries enjoys wide variation. The venous system does not contain the enzyme. ATPase activity of the aorta of the rat was not influenced by age or captivity. The aortic ATPase activity of various species of animals is presented.

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Gastric Secretion in Heidenhain Pouches Following Section of Vagus Nerves to Main Stomach. (19612)

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The cephalic phase of gastric secretion is nervous in origin and is mediated via the vagus nerves to the stomach. Apparently the gastric phase is humoral in origin and is mediated by "gastrin" which is elaborated by the mucosa of the gastric antrum. A Heidenhain pouch is a separated, vagally denervated, greater curvature, gastric pouch. Thus, the exocrine secretion from this pouch is stimulated, probably exclusively, by the humoral phases of secretion, *i.e.,* antral and intestinal phases. Any observed change in Heidenhain pouch secretion produced by sectioning the vagi to the main stomach may be presumed to reflect a proportional change in the circulating chemical substances (hormone(s) and secretagogues) which are responsible for the chemical phases of secretion.

Although it is well known that the cephalic and gastric secretory mechanisms can function independently of the other, it is not yet established whether the normal function of either is impaired by the absence of the physiological activity of the other. Grossman(1), recently stated ". . . that vagal stimuli and gastrin can act together in stimulating gastric secretion is certainly in accord with observations, in the sense that any two different stimuli can act together additively. However, the view that either of these two mechanisms for secretion regularly participates in producing a response to the other is open to serious question." Evidence is accumulating(2-4) that the gastric secretory response to vagal stimulation is decreased when the antral mucosa is defunctionalized by cocainization, vascular impairment, or excision. The conclusion has been advanced that the cephalic phase is actually neurohumoral and not solely neural as formerly held. There is much less evidence of participation of the vagi in the gastrin mechanism. To our knowledge, no quantita-

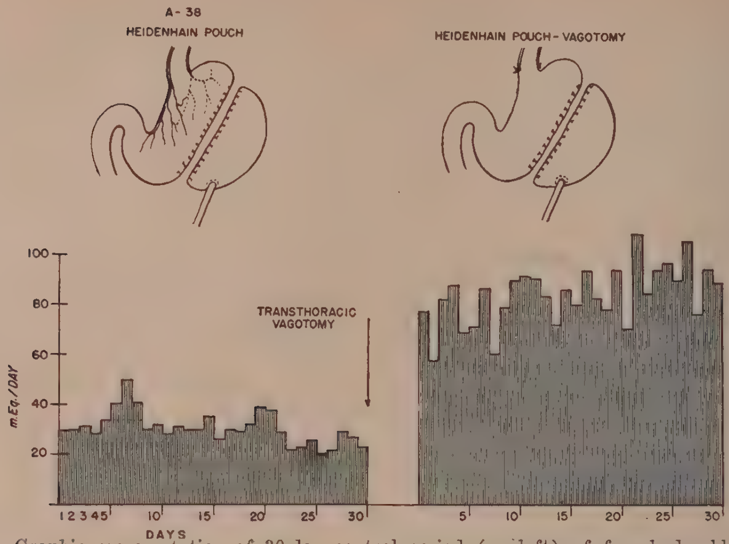

FIG. 1. Graphic representation of 30-day control period (on left) of free hydrochloric acid collection (expressed in mEq.) from Heidenhain pouch of Dog A-38 as compared to similar period (on right) after sectioning the vagus nerves to the main stomach.

tive studies have been done which shed much light on the relationship of the vagi to the physiological function of the antral mucosa. However, Harkins and Sauvage(5) showed that Banthine in doses which completely inhibited the gastric secretory response to sham feeding did not significantly change the total 24 hour secretion from Heidenhain pouches. This investigation determines only the total change in Heidenhain pouch acid secretion produced by sectioning the vagi to the main stomach and does not permit an analysis of the factors involved.

Methods. Large Heidenhain pouches, drained by a water-tight nylon cannula into football bladders for collection purposes, were made in the usual manner. After full recovery from surgery (usually two weeks) quantitative 24 hour pouch secretion collections were begun. These collections were considered as valid only if the animal maintained the following conditions: (1) constant body weight, (2) normal serum chlorides, (3) constant daily food intake. After at least 28 days of valid collections as a standard Heidenhain pouch, a transthoracic bilateral vagus section was done. After a recovery period, collections were resumed and 28 or more valid days of secretion collected according to the criteria of the control period. Barium radiographic studies of the gastric emptying time were made before and after vagus section.

Results. The accompanying illustrated graph (Fig. 1) and Table I summarize the quantitative effect of section of the vagus nerves to the main stomach on the acid secretion from Heidenhain pouches. There was a definite increase in the secretion of free hydrochloric acid as expressed in milli-equivalents per 24 hours following the vagus section. This increase in secretion was maintained throughout the 28 day test period of collection of total 24 hour samples of pouch secretion. The x-ray studies showed that the passage of barium through the stomach was markedly delayed by vagus section.

Discussion. If the vagi to the gastric antrum are important in the release of gastrin, similar to the direct effect of these nerves on the parietal cells, one would expect section of the vagus nerves to decrease such gastrin release. This possible depressant role has been overshadowed in our experiment so that section of the vagus nerves has produced the opposite effect, *i.e.*, an increase in the chemical phase of acid secretion. Following the vagus section, the time required for animals to empty a barium meal from the stomach is prolonged

TABLE I. Effect of Transthoracic Vagotomy on the Quantitative Acid Secretion from Heidenhain Pouches.

	Before, mEq.	After, mEq.	% increase
A–38	46	88	92
A–69	14	47	236
A–71	20	105	425
Avg	27	80	196

significantly. Thus, if food remains in contact with the gastric antrum for a longer period after vagus section than before, it is reasonable to assume that the antral phase of gastric secretion may be increased.

Conclusions. In the dog, section of the vagus nerves to the main stomach causes a marked increase in the quantitative 24 hour acid secretion from Heidenhain pouches.

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Intrapleurally Injected Colloidal Au^{198} and Growth and Implantation of Free Tumor Cells in Pleural Exudate.[*] (19613)

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Several authors(1) have reported clinical trials with intrapleurally injected colloidal Au^{198} in the treatment of malignant pleural effusions and thoracic neoplasms. However, to the best of our knowledge, no attempt has been made to treat similar conditions in experimental animals. Previously(2,3) we have described a method of testing the effect of radioisotopes on free tumor cells in the peritoneal fluid and on their implants in the abdominal wall. A similar method provided a tool for assaying the effect of intrapleurally injected colloidal Au^{198} on free tumor cells in pleural cavity. The results of this assay are reported below.

Material and methods. A. *Technic of tumor cell inoculation.* Requisite numbers (1 to 15 millions) of tumor cells (Sarcoma 37 in CFW mice, carcinoma in C3H and melanoma in dba)[†] were inoculated (in a volume of 0.2 to 0.5 ml) into the right phrenico-costal sinus (see details in (4)). Colloidal Au^{198} was injected in the same area a few days later. B. *Treatment.* Colloidal Au^{198}[‡] was diluted with 0.85% NaCl solution to the concentration of 0.4 mc per cc, and doses of 0.5 ml of this solution were used. For each tumor, 3 series each of 20 mice were inoculated with tumor cell suspensions. On the 5th or 6th day after inoculation, 10 mice of each series were injected intrapleurally with 0.2 mc of Au^{198}, while 10 mice were kept untreated as controls. Three or four days later the pleural exudate was withdrawn from at least 5 mice of each group and assayed as described below. On account of slow growth of melanoma cells, mice inoculated with such tumor cells were treated on the 10th day and sacrificed on the 20th day. D. *Assay of the results.* Stained specimens of the pleural exudate from treated mice and from controls were examined for morphological characteristics of tumor cells and for percentage of their mitoses. In a few animals of each group this microscopic assay of tumor cells was supplemented by biological assay: failure of inoculation of pleural exudate (0.1 ml) from treated mice to induce tumors

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[*] This work was carried out under Contract with the Division of Biology and Medicine, U. S. A. E. C.

[†] CFW mice obtained from Carworth Farms, New City, N. Y. C3H and dba obtained from Roscoe Jackson Memorial Laboratory, Bar Harbor, Me.

[‡] Colloidal Au^{198} obtained from Dr. D. L. Tabern, Abbott Laboratories, N. Chicago, Ill.

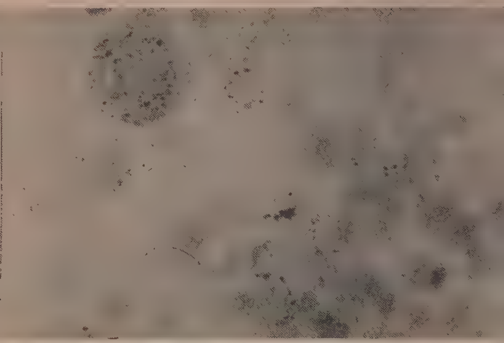

FIG. 1. Sarcoma-37 cells in pleural exudate of mouse 9 days after intrapleural inoculation of 10000000 cells. Aceto-Orcein × 600.

FIG. 2. Pleural exudate of mouse 9 days after inoculation of Sarcoma-37 cells and 4 days after intrapleural inj. of colloidal Au^{198} (.2 mc). Aceto-Orcein × 600.

in new mice was considered as evidence of nonviability of any tumor cells remaining in the exudate. Additional evidence about the effect of Au^{198} was obtained from the autopsy material. E. *Assay of Au^{198} distribution.* New CFW mice of 23 to 26 gr weight were injected intrapleurally with 0.002 to 2 mc Au^{198} and sacrificed at various intervals (see legends to Tables I and II). Their thoracic tissues as well as specimens of the liver and the spleen (for comparative study) were triturated and dried for estimation of their radioactivity. It was found that the weight of organs in sacrificed mice varied considerably, apparently on account of variations in degree of hyperemia and edema. For this reason, we used in each animal the whole amount of tissue (content of mediastinum, diaphragmatic and costal pleura), the whole organ (spleen or lung) or its fragment of certain size (one-twelfth of the liver). In order to obtain data on storage of colloidal Au^{198} in tumor tissue, similar experiments were carried out on mice bearing thoracic tumors of various strains and injected intrapleurally with doses of 0.1 mc of Au^{198}.

Results. Eight to 10 days after inoculation the peritoneal exudate withdrawn from the untreated controls contained 75 to 85% of tumor cells (S-37, S-180 or Carcinoma) 10 to 20% of which showed mitoses (Fig. 1). The transfer of this exudate (even diluted 1:10 or 1:20) into new mice resulted in their abundant multiplication. On the contrary in treated mice only scarce and cytologically abnormal tumor cells were found in the pleural exudate (Fig. 2), the amount of which was considerably lower (0.1 cc or less) than in controls (up to 0.7 cc). The cell abnormalities resembled those of free peritoneal tumor cells treated with radioisotopes(3). Transfers of these cells into new mice induced positive "takes" in only 6% of pooled results. The exudate of treated mice contained numerous macrophages loaded with tiny clumps of colloidal gold, resembling the pigment-loaded macrophages of melanoma exudate which, for this reason, were found unsuitable for study of its cellular composition in gold treated mice. The control groups each comprising 30 mice (3 groups of 10 mice for each tumor strain) showed, after 15 days only 12 survivals for S-37, 15 for S-180 and 13 for carcinoma. Tumors were found in all these animals. As a contrast, none of the treated mice died during the 15 days observation period. Only regressing nodules, sometimes consisting mostly of necrotic tissue, were found in the mediastinum of these mice. All melanoma controls survived the observation period of 30 days, at which date they were sacrificed. Large tumors, often spreading in the abdomen or in the subcutaneous tissues, were found in their pleural cavities. These tumors were absent in treated mice of the melanoma group, which carried however small perfectly round (1 or 2 mm in diameter) nodules of firm melanoma tissue.

It is obvious from distribution studies in new CFW mice (Table I) that a relatively

TABLE I. Distribution of Intrapleurally Injected Colloidal Au^{198} in Thoracic Cavity of Sacrificed Mice. Amount of radioactivity (counts/min).*

	First series (inj. dose = .1 mc†)	Second series (inj. dose = .3 mc†)
Right lung	5244 (1378-12951)	10942 (5360-22518)
Left ”	817 (238-1526)	3772 (1417-5287)
Mediastinal tissue	13924 (4084-23150)	23805 (5960-38546)
Liver fragment (1/12 of organ)	10690 (6041-14397)	27938 (12282-37563)
Spleen	2777 (1558-4043)	24972 (15221-29721)
Diaphragmatic pleura	1447 (1027-1911)	8428 (3054-13920)
Right costal ”	366 (305-428)	2472 (918-3411)

* Avg 30 mice and (in parentheses) individual extremes. Animals sacrificed 3, 7 or 14 days after inj. of Au^{198} into right pleural cavity. All specimens from same group of mice counted on same day.

† Millicurie.

large portion of radioactive material was stored in the mediastinum.

Microscopic examination of smears from mediastinal lymph nodes (Fig. 3) and from lung tissue revealed the presence of agglomerated colloid particles (described in (3) inside macrophages or in the lymph, but not inside lymphocytes. The pattern of radioactivity distribution was not modified by the use of C3H mice instead of CFW or by dilution of Au^{198} with inactive gold instead of saline solution. Moreover, it is evident from Table I that in spite of massive storage in the mediastinum of intrapleurally injected Au^{198}, the major portion of it escaped into the liver (an amount 12 times higher than that indicated in the table). The comparison of the results in two series suggested that Au^{198} distribution between the thoracic and the abdominal tissues can be modified by variations in the size of injected dose. Accordingly several groups each of 10 mice were injected with increasing doses (0.002 to 2.0 mc) of Au^{198} and sacrificed after 2 or 3 days for estimation of radioactivity in their tissues. The data of each group were used for comparison of the average radioactivity in the thoracic material (T) with that of the abdominal material (A), thus computing the ratio

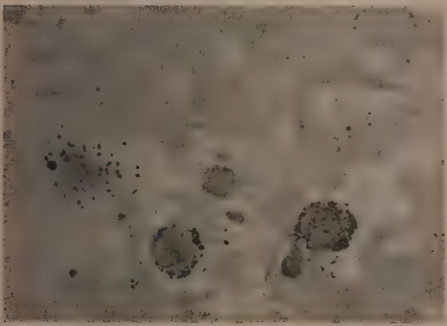

FIG. 3. Smear from a mediastinal lymph node 48 hr after intrapleural inj. of .3 mc of radioactive colloidal gold. Wright's stain × 600.

$$\frac{T}{A} = \frac{\text{mediastinum} + \text{lungs} + \text{diaphragmatic and costal pleura}}{\text{spleen} + 1/12\text{th of the liver}}$$

as an index of radioactivity distribution between thoracic and abdominal tissues. It should be emphasized that this ratio is purely conventional, since only a fragment of the liver is included in the denominator, in order to avoid large differences between numerator and denominator. The results are presented in Table II.

It is shown that even a slight increase in the dose of Au^{198} was paralleled by the decrease of the $\frac{T}{A}$ ratio *i.e.* by the increase of the portion of injected dose deposited in the abdominal organs.

In an attempt to evaluate the prospects of intrapleural Au^{198} treatment of thoracic tumors (10 to 12 days old), the radioactivity per gram tissue of these tumors was compared with the radioactivity in the equal amount of mediastinal tissue (M) or liver (H) in the same mouse. The ratios $\frac{T}{M}$ and $\frac{T}{H}$ were calculated for 3 groups of 10 mice, each group bearing different tumors. The results varied considerably for various tumor strains: for Carcinoma $\frac{T}{M}$ was 0.61 and $\frac{T}{H}$ –0.61; for Melanoma $\frac{T}{M}$ –0.36 and $\frac{T}{H}$ –0.85, but for

TABLE II. Influence of Dose of Intrapleurally Injected Colloidal Au^{198} on Its Distribution Between Thoracic and Abdominal Tissues (Ratio $\frac{T}{A}$).

Dose inj., mc	Ratio $\frac{T}{A}$
.002	2.2
.005	1.5
.02	1.2
.05	1.17
.1	.98
.3	.93
.5	.53
2	.35

Samples of thoracic tissues comprised both lungs, mediastinal content, costal and diaphragmatic pleura; samples of abdominal tissue—fragment of 1/12 of liver and whole spleen. Each ratio is computed from averages in 10 mice, sacrificed 2 or 3 days after injection.

S-37 – $\frac{T}{M}$ was 2.37 and $\frac{T}{H}$ –2.95. Considering that intrapleurally injected colloidal Au^{198} is stored mainly in the mediastinum and in the liver, the portion of this isotope intercepted by tumor tissue is significant.

Discussion. We have used peritoneal and pleural exudates as growth media and peritoneal and pleural cavities as incubators to grow tumor cells of various strains as free cells and to study the effect of Au^{198} on these cells. We have found in both fluids the same essential signs of damage to the tumor cells by radiation: distortion of mitotic figures and loss of viability followed by disintegration of cell structure. These findings agree with the observations by Haddow(5) and Koller(6) on the close association of mass appearance of abnormal mitoses in tumor tissue with the loss of its viability, as the effect of radiation or of nitrogen mustards.

Free tumor cells were affected with small doses of Au^{198} (0.2 mc) more extensively in the pleural exudate than in the peritoneal fluid (as reported in(3)). This difference may be accounted for by the much smaller volume of pleural exudate and smaller dimensions of the pleural cavity. Obviously the concentration of the injected dose was higher in the pleural exudate and the ratio "amount of radiation per unit surface" was considerably higher than in the peritoneal cavity. In our current experiments we attempt to study separately, on quantitative lines, the direct (tumor cell degeneration) and the indirect (atrophy of the tumor bed) inhibiting effect of radiation on the tumor growth.

It is known(1,3) that colloidal Au^{198} is taken up from body fluids by macrophages, lymph nodes and lymphatics. These tissues are abundant in the mediastinum which therefore is the center of colloidal Au^{198} storage in the thoracic cavity. A lesser site of storage is found in the lungs. Since no marked changes of the distribution pattern were found in animals sacrificed at various intervals after injection, it is evident that the bulk of colloidal Au^{198} reached its ultimate destination within 3 days after its intrapleural administration.

Our results on the distribution of intrapleurally injected gold colloid agree closely with the data on the pattern of absorption of other types of particulate material (carmine, lampblack) from the pleural cavity of the dog(7,8).

Tables I and II show that reduction of the dose did not prevent the escape into the liver and spleen of the major portion of intrapleurally injected gold, but it changed the ratio between the thoracic and the abdominal storage of gold in favor of the former. Since the complications of treatment with colloidal Au^{198} result from extensive damage to the liver and the spleen by relatively high doses of radioactivity, it seems reasonable to avoid or to minimize this damage and to deposit, at the same time, sufficient amounts of Au^{198} in the thoracic cavity by using repeated low doses rather than a single massive dose.

Summary and conclusions. (1) Mice inoculated intrapleurally with suspensions of free tumor cells of various strains (S-37, S-180 in CFW mice, carcinoma in C3H mice, Melanoma in dba) were treated 4 or 5 days later, by intrapleural injections of colloidal Au^{198} (0.2 mc). After 3 or 4 days, specimens of pleural exudate were withdrawn, stained and compared, as for cellular composition, with those of untreated controls. (2) It was found that in all controls the pleural exudate contained numerous growing (mitoses) tumor cells. Their transfer (intrapleural or intraperitoneal) into normal mice resulted in renewed vigorous growth. However, in treated mice tumor cells were scanty, abnormally shaped, with few and abnormal mitoses. They were non-viable as demonstrated by their failure to grow after transfer into new mice. Moreover, the exudate of treated mice contained numerous macrophages loaded with clumps of colloidal gold(3). In untreated controls the growth of free tumor cells in the exudate resulted, as a rule, in their implantation into mediastinum and later on in abundant growth of these implants. Only small nodules, often consisting of necrotic tissue, were found in treated animals. (3) Intrapleurally injected colloidal Au^{198} was largely stored in the thoracic cavity (in macrophages and in lymphatic tissue), mainly in the mediastinum, but nevertheless it reached the liver and the spleen in considerable amounts. However, it was found that ratio $\frac{T}{A}$ (index of Au^{198} distribution between thoracic and abdominal tissues) could be increased by the use of small doses for intrapleural injection of Au^{198}(5). Infiltration of thoracic tumors with intrapleurally injected Au^{198} was noted. (4) It is concluded that irradiation with intrapleurally injected Au^{198} induced degeneration and disintegration of free tumor cells in the pleural exudate. It is presumed that inhibition of tumor cell implantation was the result of radiation damage to implanted tumor cells (direct effect) and to the tumor bed (indirect effect on tumor growth).

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Pulmonary Edema in Oxygen Poisoning.* (19614)

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In the majority of investigations on pulmonary edema associated with oxygen poisoning, the criteria for the evaluation of the degree of the edema have consisted of gross and microscopic examination of the lungs or lung sections removed at autopsy(1-3). The gross examination has consisted of an approximate estimate of the size and texture of the extirpated lungs, the color, the presence of foam in the airways and the determination of whether or not fluid exudes from a cut surface. In order to supplement this usual pathological examination and to provide quantitative numerical data suitable for statistical evaluation, a method of lung analysis has been devised which will evaluate factors useful for determining the degree of pulmonary edema (4). This new method, which consists of a chemical and physical analysis of lungs extirpated at necropsy, has been used to determine quantitatively the degree of pulmonary edema following exposure to pure oxygen at ground level barometric pressure.

Methods. A group of 7 to 9 healthy guinea pigs of approximately the same size were selected and placed on the same dietary regime in the same laboratory for a period of 2 to 3 weeks. At the end of this period, 2 animals (controls) were killed and their lungs ana-

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TABLE I. Changes in Means and Stand. Dev. of Lung Measurements during O_2 Poisoning.

Duration in O_2, hr	No. of animals	L/B, g/mg	L/IPN, g/mg	SPN/IPN, g/mg	Hb/IPN, g/mg
0 (controls)	9	7.15 ± 1.15	.113 ± .017	.91 ± .05	2.47 ± .54
36–60	5	8.61† ± 1.03	.127* ± .018	.99* ± .24	2.82* ± 1.21
60–83	10	14 ± 5.9	.186 ± .056	1.71 ± .65	1.92* ± .60
84–108	10	16.1 ± 4.4	.198 ± .076	1.71 ± .65	3.25* ± 3.5
108–132	4	19 ± 5.3	.181 ± .031	1.45 ± .44	2.65* ± .44

* Not significantly different from control.

† Significantly different from control at 10% level; all other values significantly different from control at less than 1% probability.

lyzed. The remaining animals of the group were placed in an "oil-drum" type sealed gas chamber containing soda lime racks, and into this chamber a stream of pure oxygen was passed through an inlet tube. An outlet tube permitted escape of chamber gas which was periodically analyzed for oxygen and carbon dioxide. After 24 to 48 hours, one or more guinea pigs were removed, sacrificed and their lungs analyzed. During the succeeding 3 or 4 days the remaining animals were removed, with no animal remaining more than 6 days in the oxygen atmosphere. After 4 days in oxygen the animals commenced to die and any dead animals were rejected. After removal from the chamber, the animals were immediately and quickly killed by guillotine, the chest opened and the lungs removed. This operation required about 2 to 3 minutes. The procedure for lung analysis has been described previously(4), as well as values for a large series of normal guinea pigs(5). After removal and trimming of the lungs, they were weighed. The lungs were then ground to a pulp in sand and extracted until colorless with 1% NaCl solution. The insoluble residue was analyzed by the Kjeldahl procedure for nitrogen, and this fraction, which consists of nitrogen in the insoluble protein of lung framework, pulmonary vessels and parenchyma, is designated as the insoluble protein nitrogen fraction. The saline extract contains the soluble protein, including hemoglobin. An aliquot of the extract was dried and the dried residue extracted with an ether-alcohol-acetic acid mixture, which provides a clear solution of acid hematin. Another aliquot of the saline extract is treated with tungstic acid to precipitate protein which is determined by the Kjeldahl procedure. The soluble protein, other than hemoglobin, is determined by difference. In one group of guinea pigs the lungs were removed and sections prepared for histological examination. The sections were prepared either by staining with aniline blue and chromotrope for erythrocytes or by treatment with periodic acid and staining with Schiff's reagent, which reveals acidophilic fibrinoid material.

Terminology. The following abbreviations will be used. The lung weight measured in g will be designated as L, and the body weight (kg) designated as B. The lung weight:body weight ratio is L/B. The insoluble protein nitrogen fraction (mg) is IPN, and the soluble protein nitrogen fraction (mg), SPN. Pulmonary hemoglobin (mg) is designated as Hb.

Results. Results of the analyses are given in Table I. The animals have been arranged in groups according to the duration time in oxygen, which is given in the first column. The values in the table are the means and standard deviations for each group. The first row of numerical values in the table contains the control data for the animals not exposed to oxygen. For the animals exposed to oxygen, the significance of variation from the control values has been computed, using the method described by Snedecor(6). Fig. 1 contains the individual values of L/B, and Fig. 2 the values of L/IPN from 6 experiments, each of which used 7 to 9 guinea pigs. These figures reveal the variability in the congestive edema which is particularly noticeable after more than 48 hours of pure oxygen breathing.

Discussion of results. After 48 hours of exposure to oxygen, symptoms of pulmonary edema commence, and the condition increases progressively until death occurs after 4 to 6

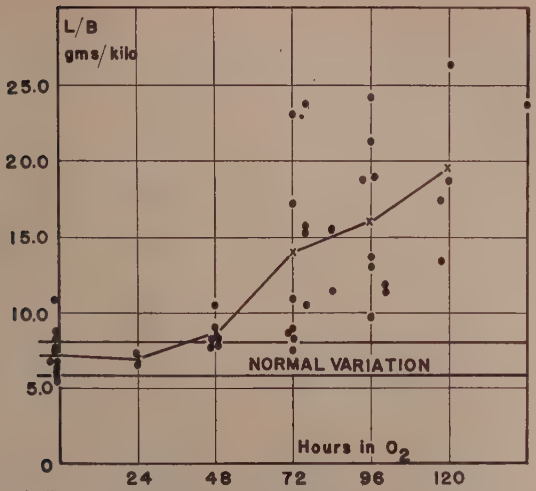

FIG. 1. Variation of (lung wt)/(body wt) ratio after exposure to pure oxygen for varying exposure duration times.

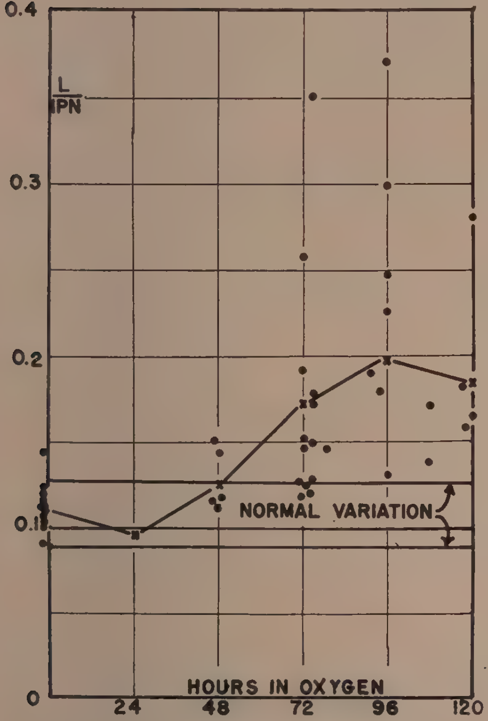

FIG. 2. Variation of (lung wt)/(insoluble protein nitrogen) ratio after exposure to pure oxygen for varying exposure times.

days of pure oxygen breathing. The lungs increase in size with the lung weight/body weight ratio more than doubling. There is considerable variability between values, but the changes are highly significant. The fraction IPN is a fraction which does not change appreciably with the onset of acute edema. The insoluble protein nitrogen contains the protein nitrogen of the lung framework and parenchyma. If acute congestive edema is considered as an invasion of pulmonary tissue by fluid containing plasma protein and hemoglobin, then the IPN fraction will not increase, while SPN and Hb should increase. It has been shown previously(5) that there is close correlation between lung weight and insoluble protein nitrogen of normal guinea pigs; hence the IPN fraction can be used as an estimate of the size of the non-edematous lung, and from relations previously established for normal guinea pigs, the predicted normal values of both SPN and Hb can be obtained. If L/IPN increases above normal, this value can be used as a quantitative measure of the edematous process and can be considered as an estimate of the increase in lung weight. SPN/IPN can be considered as a measure of the increase of soluble protein in the edematous lung. Likewise, an increase in Hb/IPN will indicate quantitatively the increase in pulmonary hemoglobin and will be a measure of the pulmonary "congestion." It is to be noted that the ratio L/IPN increases significantly after 60 hours of oxygen breathing from a control value of 0.113 to a value of 0.198, an increase in the ratio of approximately 75%. The increase in SPN/IPN from the control value of 0.91 to 1.71 represents an increase of soluble protein in the lung to a value almost double the normal value.

Although most observers report "bloody lungs" as a result of oxygen poisoning, the results obtained in this present study do not indicate a pulmonary hemoglobin significantly higher than normal. The mean values of pulmonary hemoglobin in 3 of the 4 experimental groups increase, but the variability, both in controls and in oxygen poisoning groups, is so great that the changes are without significance. In one group, namely, the group subjected to pure oxygen for 60 to 83 hours, the Hb/IPN value was 1.92, this being lower than the control mean of 2.47.

Histological examination of the sections stained for erythrocytes revealed in some cases extensive invasion of the alveoli and interstitial spaces with erythrocytes. This would indicate that in some lungs or parts of lungs there is extensive congestion. When, however, the entire lung is analyzed, the pulmonary hemoglobin is not significantly raised. This would lead to a conclusion, supported also by visual examination of the lungs, that congestion was not uniform throughout the lungs, but localized in regions. An interesting observation also noted by Karsner(2) and Barach(7) was the finding of an increase in "fibrinoid" material after oxygen poisoning.

Conclusion. When guinea pigs are placed in an environment of 95 to 99% oxygen at ground level barometric pressure 73 to 75 cm Hg, the animals will develop pulmonary edema as a symptom of oxygen poisoning after 48 hours. The condition becomes progressively worse, and the animals die after 4 to 6 days in the oxygen atmosphere. Lung analyses during severe oxygen poisoning indicate an approximate doubling in lung weight and soluble protein nitrogen. Pulmonary hemoglobin, while quite variable in amount in different lungs, does not increase significantly in the entire lung during oxygen poisoning, but local regions, as shown by histological examination, may be intensely congested.

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Uptake of Radioactive Sulfur by Chondrosarcomas in Man. (19615)

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The ground substance of cartilage contains sulfuric esters of polysaccharides. Dziewiatkowski has shown that the radioactive sulfur of labeled sulfate given to rats is fixed by cartilage(1) and incorporated into chrondoitin sulfuric acid(2). The other tissues of the rat retain S-35 in smaller amounts and for shorter periods(3). There is little exchange between inorganic sulfur and the sulfur of amino acids and proteins(4), so that the major portion of the labeled sulfate given to rats is excreted within 24 hours(3). In man the excretion is somewhat slower(5). It is known that some differentiated tumors have metabolic patterns similar to those of the tissue of origin. Metachromatic staining properties indicate specifically the presence of sulfuric esters of polysaccharides(6) in the ground substance of chondrosarcomas, as well as in that of cartilage. On the basis of these facts it appeared likely that radioactive sulfur given as sulfate to man would be taken up selectively by neoplastic as well as by normal cartilage. Two cases of chondrosarcoma offered an opportunity to verify this assumption. Sulfur-35 has seldom been used in man, and only in small amounts(5,7), presumably on account of its relatively long physical half-life (87 days). Recently Walser gave 100 μc of S-35 sulfate to measure the extracellular fluid of normal human subjects(8).

The uptake of S-35 by tissue cultures of human cartilage and tumors was studied by Layton(9), who pointed out the feasibility of confirming the cartilaginous nature of the growths by this test *in vitro*. However, the histologic nature of the explants was not clearly ascertained. The cultures of the cartilaginous tumors studied (osteochondroma and osteochondrogenic tumor of osteoblasts and giant cells) appeared to fix about as much sulfur as the cultures of bone or cartilage invaded by other tumors.

Experimental. Radioactive sodium sulfate was prepared by neutralizing with sodium hydroxide the carrier free H_2 S-35 O_4 supplied by the Isotopes Division of the A.E.C., Oak Ridge, Tenn. It was dissolved to about 1 mc per ml in pyrogen free distilled water with 2.2 or 3.3 mg of sodium sulfate per mc as carrier. The solution was autoclaved and given in a single injection through the tubing of an intravenous drip of 5% glucose. The first patient (H., 26-year male) had a rapidly growing chondrosarcoma of the ilium partly resected by a previous hemipelvectomy. Local extension to the sacrum and to the soft tissues and metastasis to the lungs were known to be present. The patient was given 6.7 millicuries of S-35 sodium sulfate. Tissue samples were obtained by biopsy 65 hours later. The patient expired 11 days after the injection. Necropsy disclosed metastasis to the lungs, pleurae, liver, and lymph nodes, and neoplastic invasion of pulmonary arteries. The tumor consisted of round or elongated neoplastic cartilage cells within a variable amount of ground substance; in places the cells were closely grouped and resembled an undifferentiated sarcoma. The second patient (G., 41-year male) had in the head of the tibia a slower growing tumor that had been repeatedly curetted. The leg was amputated 50 hours after injection of 2.9 millicuries of sodium sulfate. The chondrosarcoma had invaded the soft tissues. It was composed of neoplastic cartilage cells isolated within abundant ground substance. The patient was apparently well 3½ months after amputation. Numerous clinical tests in both cases disclosed no signs of toxicity referable to the radioactivity of the sulfur. Direct radiation counts were made with a 1.8 mg/sq cm mica end-window Geiger-Mueller tube adequately protected and applied against the cut surfaces of the tissues of both patients. Samples of tissues kept in the frozen state were analyzed in duplicate for their S-35 content. They were hydrolyzed with 1 ml of 10% sodium hydroxide. After addition of 5 ml of a 0.35% sodium sulfate solution as carrier, they were oxidized with 5 ml of Denis' reagent(10). The melt was dissolved with about 10 ml of 5% hydrochloric acid, and the sulfate was precipitated at pH 2 to 3 with 5 ml of 5% barium chloride. The activity of the barium sulfate was measured in metal cups with a G-M tube (1.8 mg/sq cm mica window) and with a Tracerlab autoscaler. Individual samples were counted for at least a total of 4096 counts to insure a standard counting error of ±1.6%. The net counts were corrected for self-absorption to a standard weight of barium sulfate. In a typical experiment the recovery of S-35 added as sodium sulfate was 79 and 83%. The activity in each sample was referred to the wet weight of the tissue; it was compared to the activity of aliquots of the injected solution of S-35 simultaneously treated by the same methods.

Results. To permit a direct comparison of the concentrations of S-35 found in the tissues of different patients, they were referred to the injected doses of S-35 calculated on a body-weight basis. The "relative concentrations" of radioactive sulfur given in the charts and table are expressed as percents of the theoretical concentration that would have occurred if the injected isotope had been evenly distributed throughout the body and completely

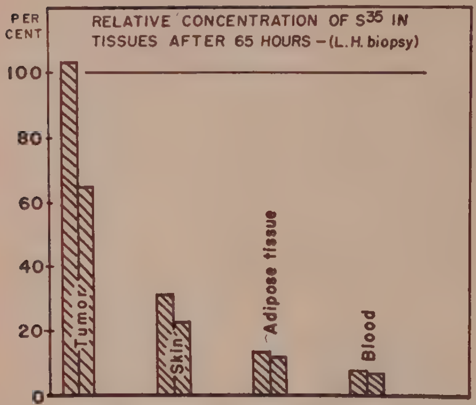

FIG. 1. The 100% relative concentration (initial avg body level) corresponds to .124 μc of S-35 per g wet wt of tissue on day of inj. decayed to .121 μc per g on day of biopsy.

retained ("initial average body level").

In patient H., 65 hours after the injection (Fig. 1), the concentration of S-35 in a subcutaneous nodule of chondrosarcoma was about 3 times as high as in the skin (epidermis and upper dermis), 6 times as high as in the subcutaneous adipose tissue, and 11 times as high as in the blood. At the death of this patient (Fig. 2), samples of both primary and metastatic chondrosarcomas contained large amounts of S-35. The concentration varied markedly between samples of tumors of different sites; even in adjacent samples of the same tumor the variation appeared greater than in adjacent samples of normal tissues. The average concentration of S-35 in normal cartilage was about two-thirds the average concentration in the neoplasm. Progressively lower levels were found in bone marrow, spleen, kidney, skin, liver, cortical bone, adipose tissue, and voluntary muscle. In the muscle the concentration of S-35 was about one two-hundredth that in the tumor. The ratio of concentration between tumor and soft tissues had increased with time, since the isotope level was about the same in the chondrosarcoma, and had rapidly fallen in the skin and adipose tissue. There was a similar fall of the level of S-35 in the blood (Table I).

The relative concentrations of radioactive sulfur in the tissues of the leg of patient G., amputated 50 hours after injection, are given in Fig. 3. In the skin and blood the relative concentrations were about the same as in the specimens of the first patient taken 65 hours

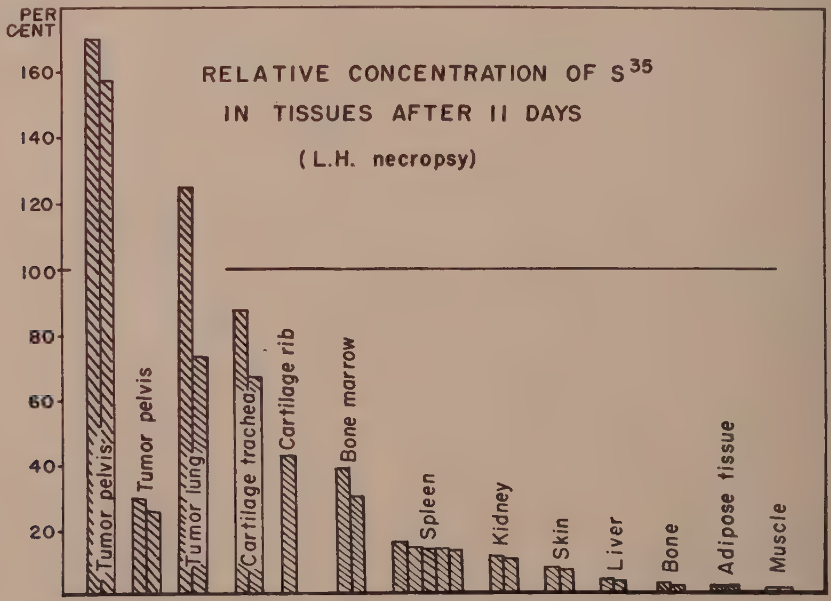

FIG. 2. The 100% relative concentration corresponds to .124 μc per g on day of inj. decayed to .114 μc per g at time of death.

TABLE I. Relative Concentration of S-35 in Blood of Patient H.

Hr after inj.	% conc.
12	61.7
65	7.4
180	1.4

after injection. The lower relative concentration in the adipose tissue of G. seems related to the better nutritional status and higher fat content. If the 2-day specimens of G. and the 11-day specimens of H. are compared, the expected fall of concentration is noted in the bone and muscle; on the other hand the similarity of level of S-35 in the specimens of cartilage suggest that in man, as in rats(2), sulfur is retained over long periods in cartilaginous tissues. In the chondrosarcoma of G., the relative concentration of S-35 was similar or higher than in the tumor of the first patient, reaching 2.5 times the theoretical initial average body level. The concentration in the chondrosarcoma averaged 4.4 times that present in the cartilage and 63 times that in voluntary muscle.

Discussion. The high but variable uptake of sulfate sulfur by the chondrosarcomas seems related to the amount of ground substance and to the proliferative activity of the cells. The concentration was maximal in the tumor of G. that had abundant ground substance.

Radioautographs of biopsy specimens of the patient H. (Fig. 4) disclosed larger amounts of isotope in the portions of the tumor where the neoplastic cells were interspersed in ground substance. However, all viable portions of the growth, even the undifferentiated sarcoma-like areas, contained more S-35 than the soft tissues. The concentration was generally higher at the growing edges of the tumor and minimal in necrotic portions. Small amounts of S-35 were taken up by the epidermis and by the fibrous tissue. Somewhat larger amounts were visualized in the probably newly formed dense connective tissue adjacent to the tumor or near the blood vessels supplying it. Labeled sulfate is probably incorporated, although to a lesser degree than in cartilage, in the intercellular substance of connective tissue, as indicated by the studies of Layton(11).

The observation that radioactive sulfate specifically localizes in chondrosarcomas suggests some possible diagnostic or therapeutic applications.

The counts on the exposed surfaces of the surgical or necropsy specimens of both patients grossly paralleled the counts on the purified sulfur fraction of the corresponding tissues. The Geiger-Mueller counter appeared more reliable than the gross appearance in differentiating between neoplastic nodules and scar tissue at the periphery of the curetted chondrosarcoma of G., as shown by subsequent microscopic examination. However, such a diagnostic application would be limited to the direct exploration of an operative field, possibly with a probe-shaped G-M tube, to

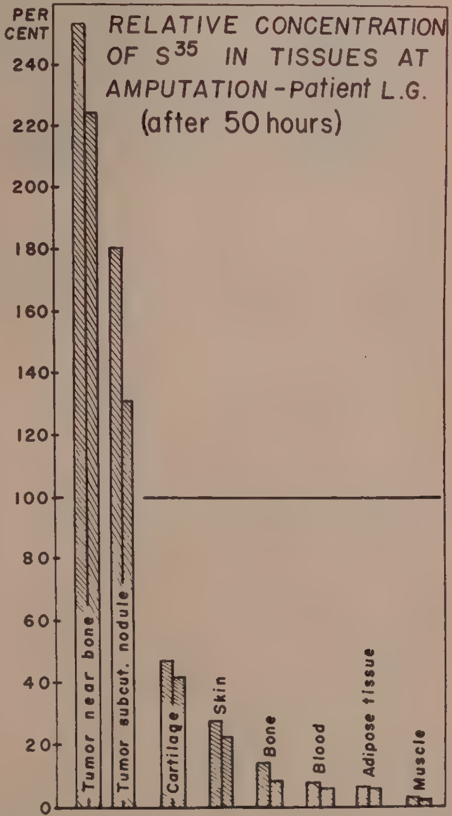

FIG. 3. The 100% relative concentration corresponds to .053 μc per g on day of inj. decayed to .052 μc per g at time of amputation.

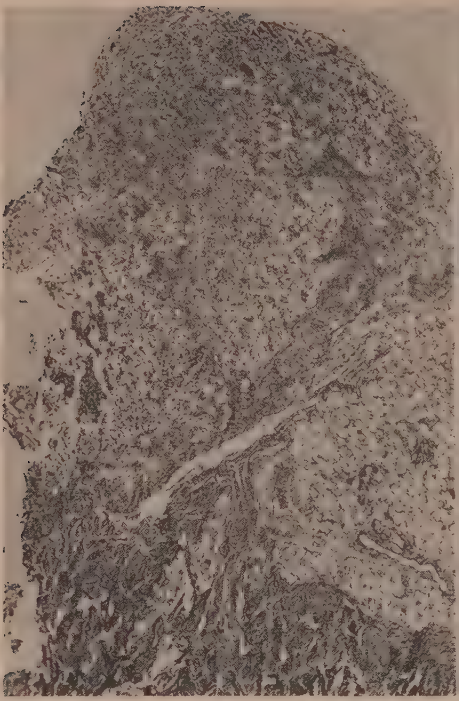

FIG. 4a (left). Subcutaneous nodule of chondrosarcoma obtained at biopsy from patient H. Neoplastic cells infiltrate the capsule (center). In lower and right fields fibrous and adipose tissues. Acid formalin fixation, paraffin embedding, 5 μ section, H. E. stain. × 42.

FIG. 4b (right). Radioautograph obtained from the section of Fig. 4a (Kodak Contrast Process Ortho film was held at about 20 μ from the surface of the unstained deparaffinized section, 97 days exposure). S-35 is demonstrated throughout the tumor, mainly in areas with abundant ground substance and at the growing edge. The dark areas in the right lower corner correspond to blood vessels. × 42.

determine the extent of a chondrosarcoma, since the soft beta rays of S-35 are absorbed by less than 1 mm of tissue.

Because of the low average energy of the radiation (0.055 MEV) and the rapid excretion of the sulfate, the doses of S-35 used in these studies can be considered to be tracer doses. Tissues with a 100% relative concentration of S-35 would have received initially 0.41 rep/day in the case of H., and 0.18 rep/day in the case of G. (calculated by formula 3 of Evans(12)).

The administration of very large doses of S-35 would be required to damage chondrosarcoma cells. Whether such doses could be tolerated by the human organism will depend not only upon the concentration in the different tissues, but also upon the time the radioisotope is retained in the respective tissue (effective half-life), and upon the radiosensitivity of the tissue. The ionization due to S-35 is limited to the tissue where it concentrates because of the very short range of the radiation. S-35 is retained at high levels in both primary and metastatic chondrosarcomas, mainly in the portions with abundant ground substance and in the areas actively growing. Large doses might, however, be harmful to normal cartilage, where they are retained for long periods, and to the bone marrow, which has a high radiation sensitivity. Recent results also indicate that very large amounts of radiation are delivered to the excretory organs immediately after administration of radioactive sulfur.

These toxic effects may outweigh the possible beneficial effects on the tumor. Additional studies on the radiation toxicity of S-35 are needed in order to evaluate whether any therapeutic benefit could be hoped for from the administration of radioactive sulfur in selected cases of chondrosarcoma.

Summary. Two patients with chondrosarcomas received respectively 6.7 and 2.9 millicuries of S-35 as sodium sulfate. Tissue samples were obtained at operations and at necropsy several days after the injection and were analyzed for S-35 content. The highest concentrations of isotope were found in the chondrosarcomas. Progressively smaller amounts were found in other tissues in the sequence: cartilage, bone marrow, spleen, kidney, skin, liver, bone, adipose tissue, and muscle. Radioautographs indicated maximum concentration in the growing portions of the chondrosarcoma and in those containing abundant ground substance. The findings are discussed in relation to the possibility of diagnostic or therapeutic applications.

We wish to express our gratitude to Mrs. Rosemary P. Mudd and to Miss Fern A. Garrett for their skillful technical assistance. We are indebted to Dr. J. H. Gast for his helpful advice, and to Dr. A. Brodsky and Dr. B. F. Boylston, of the Department of Orthopedic Surgery, for their cooperation.

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Serum Citric Acid in Hodgkin's Disease, Lymphoma and Carcinoma.* (19616)

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Thunberg(1) published an enzymatic method for quantitating serum citric acid. Benni, Schersten and Ostberg(2), employing this method, reported normal serum values of 1.5 to 3.75 mg per 100 ml of serum. Schersten (3) noted hypocitricemia in some patients with cancer. Ostberg(4) tested sera for citric acid in 8 patients with malignant disease and found values to be normal, below normal, or above normal.

Since we were unable to find any values on citric acid levels in persons with Hodgkin's disease, we undertook to make determinations of serum citric acid in 50 patients with this disease and, in addition, in 19 patients with cancer, 10 with lymphosarcoma, and in 7 with leukemia. The control group embraced 37 normal persons and 60 patients with various non-malignant diseases. In the 67 patients with Hodgkin's disease, lymphosarcoma, and leukemia 137 serum determinations were made. In 34 subjects single determinations were done, and in each of the remaining 33 persons, 2 to 5 determinations were made over a period of 4 months. The study covered a period of one year. In the Hodgkin's group data were obtained on patients during relapse and remission, in the early and terminal stages of the disease, and in mild and severe forms

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* Supported in part by grants from the American Cancer Society and the Damon Runyon Memorial Fund.

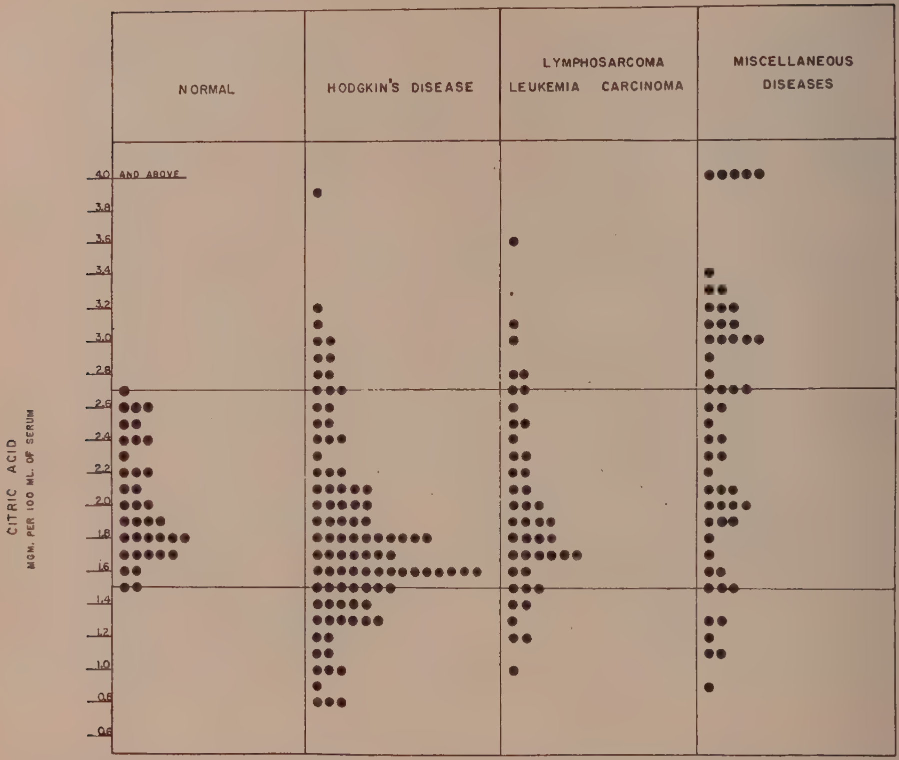

FIG. 1. Serum citric acid in normal persons and patients with a variety of disease. Each dot along the coordinate axes represents a single citric acid determination.

of the disease. In most instances blood was taken after an overnight fast and tested the same day. The procedure employed was a pentabromoacetone method as modified by Natelson and associates(5). Readings were made on the Model DU Beckman spectrophotometer.

Results. Values (Fig. 1) for the presumably healthy persons were found to range from 1.5 to 2.7 mg per 100 cc of serum; the median value was 1.95, and the mean value was 2 ± .04. Values for persons with disease varied, but no disease, whether malignant or nonmalignant, was associated with a characteristic level; levels were obtained within, above, and below the normal range, the deviation being from .8 to 9.20 mg per 100 ml. The levels may vary at different times. They may be within normal, below normal to normal, persistently high or persistently low, for the same patient.

Discussion. Our studies confirm the findings of earlier workers concerning the small quantity and narrow range of fluctuation of citric acid in the blood serum of normal persons. Values obtained by the pentabromoacetone method are approximately 75% of those obtained by the Thunberg method. This is due to the fact, as pointed out by Martensson(6), that the chemical method quantitates citric acid alone, whereas the enzymatic method quantitates three acids in equilibrium: citric, isocitric and cisaconitic.

Like Ostberg(7) and predecessors, we found that in disease citric acid in the serum may rise above or fall below the normal range, and that these changes are not connected with any specific disease or type of disease but seem, rather, to be a manifestation of deranged basic physiological mechanisms (disturbed acid base balance, renal and liver dysfunction). Ostberg, for example, demonstrated that alteration in the acid base balance of the blood produced a deviation from normal in the citric acid of the serum. Martensson(6) showed that citric acid is normally oxidized by the kidneys and, therefore, any change in kidney function from whatever cause could affect the level of citric acid in the serum. Thunberg (8) and Sjöström(9) found elevated levels of citric acid during hepatitis. We, however, did not do plasma electrolyte studies nor liver or kidney function tests on our subjects, and hence cannot relate abnormal serum citric acid levels to dysfunction of these organs.

Summary. The level of citric acid in the serum was determined in 50 patients with Hodgkin's disease, 10 with lymphosarcoma, 7 with leukemia, and in 19 with various forms of carcinoma. Sixty patients with a variety of non-malignant disease and 37 normal persons served as controls. No disease, malignant or non-malignant, was associated with a characteristic value, though variations from normal occurred in all disease categories.

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Cortisone Effect on Pneumonitis Produced in Mice by Exposure to a High Oxygen Atmosphere.* (19617)

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Application of cortisone and adrenocorticotropic hormones to the treatment of such pulmonary diseases as pneumonia(1) and bronchial asthma has focussed interest upon its effect on the inflammatory responses of the lung. The adverse effects of cortisone on experimental tuberculous disease have been reported(2) and have since been confirmed by reports of similar effects in clinical cases(3). However, since the pulmonary responses to tuberculous infection differ so markedly from those induced by other inflammatory diseases, it seemed desirable to investigate the effects of cortisone on acute pneumonitis.

Bacterial pneumonia was avoided in this study in order to eliminate the complicating factors of inhibition by cortisone of the usual host responses to infections(4). A fatal acute pneumonitis has been induced regularly in animals by prolonged continuous residence in atmospheres of 100% oxygen(5,6).

Materials and methods. In all experiments, the mice used were of a heterologous albino and Bartonella free strain, examined bacteriologically for freedom from enteric and respiratory infections. They were 8 to 10 weeks of age and averaged about 25 g in weight. Each group contained an equal number of males and females. For continuous maintenance of a 100% oxygen atmosphere, a special chamber was constructed. The bottom of the chamber was divided into compartments to permit the separation of the groups of mice. One compartment was used for the storage of the drugs and instruments needed for the experiment. The top was made of a transparent plastic material to permit observation of the animals, and was provided with a gasket so that airtight closure was possible. Two tubes were inserted into opposite ends of the chamber, one

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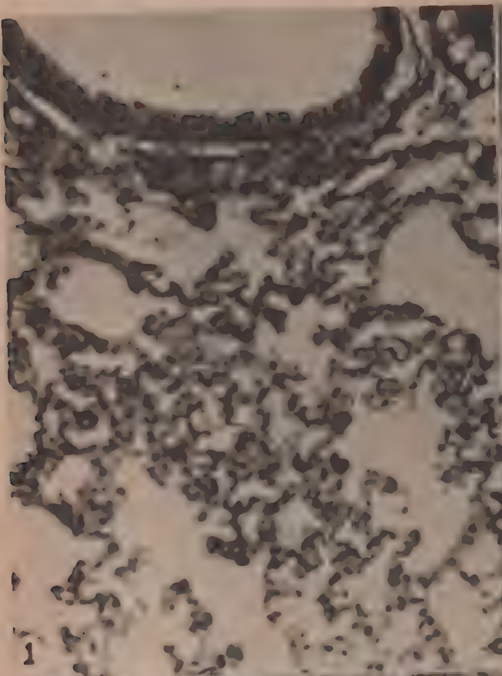
* Aided by a grant from the National Institutes of Health, U. S. Public Health Service.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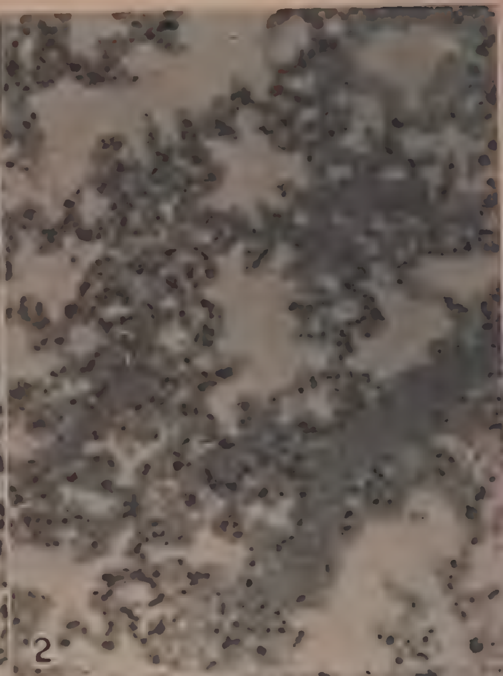
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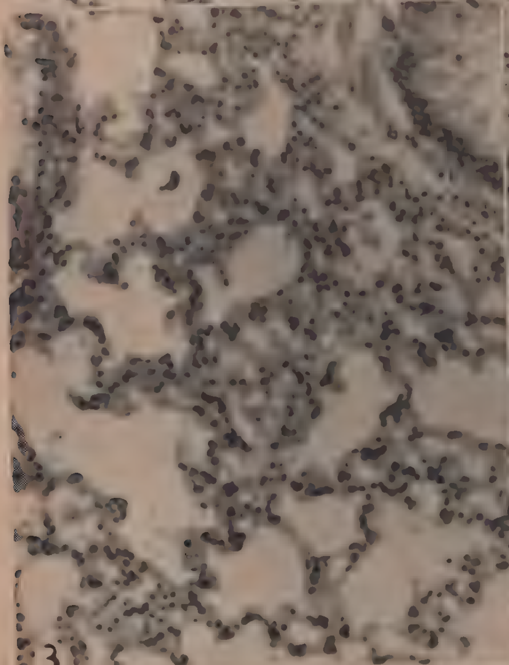
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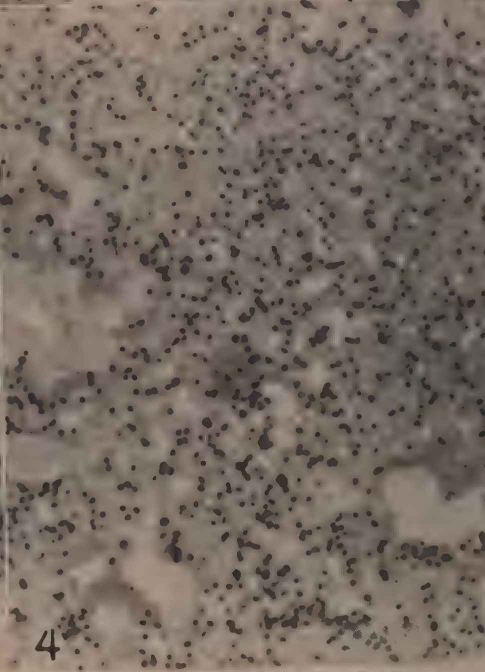
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FIG. 1. Photomicrograph of lung of control sacrificed after [illegible] days in 100% O_2, showing a relatively normal appearance. Mag. × 350.

FIG. 2. Photomicrograph of lung of cortisone treated animal sacrificed after [illegible] days in 100%

O_2 showing intense engorgement of alveolar capillaries. Mag. × 350.

FIG. 3. Photomicrograph of lung of control sacrificed after 6 days in 100% O_2, showing engorgement of alveolar capillaries. Mag. × 350.

FIG. 4. Photomicrograph of lung of cortisone treated animal that died after 6 days in 100% O_2 showing alveolar exudate, fibrin, hyaline membranes and occasional leukocytes. Mag. × 350.

through which oxygen was supplied and the other through which it overflowed. Long-sleeved rubber gloves were sealed into portholes on two sides to permit handling and injection of the animals without opening the chamber. The animals were placed in the chamber together with supplies of food and water sufficient for the duration of the experiment. The drugs and the syringes and needles necessary for their administration were also placed in the chamber. The top was sealed and 100% oxygen was introduced at a high rate of flow for a time sufficient to purge the interior of the chamber of nitrogen. The oxygen flow was then reduced and then maintained at 5 liters per minute for the duration of the experiment. The constant overflow provided the means of removing carbon dioxide. Samples of the chamber atmosphere were tested twice daily to assure maintenance of the oxygen concentration above 99%.

In the first experiment, 32 mice were given a daily injection of 1 mg of cortisone while 20 additional mice received daily injections of identical amounts of the sterile diluent used to suspend the cortisone. These injections were continued for the duration of the experiment. On the fifth day, 20 of the cortisone treated animals and all of the control group were placed into the chambers and maintained in an atmosphere of 100% oxygen. The remaining 12 cortisone treated mice were kept in cages open to room air.

Results. The results of this experiment are presented in Table I. It will be noted that the cortisone treated animals proved to be much more susceptible to the lethal effects of prolonged exposure to a 100% oxygen atmosphere, all but one of this group having succumbed before the first deaths occurred among the controls. The prolonged survival of the cortisone treated animals maintained in room air is evidence that the increased rate of mortality cannot be attributed to the cortisone alone.

TABLE I. Effects of Cortisone on Survival of Mice Continuously Exposed to 100% Oxygen.

		No. dead		
Day of exp.	Days in 100% O_2	Cortisone treated	Controls	Air controls: Cortisone treated and maintained in room air
4	—	—	—	—
5	1	3	—	—
6	2	5	—	—
7	3	11	—	—
8	4	—	—	—
9	5	—	2	—
10	6	1	10	—
11	7	—	6	—
12	—	—	2	2
13	—	—	—	1
14	—	—	—	—
15	—	—	—	—

TABLE II. Effects of Cortisone on Survival of Mice Continuously Exposed to 100% O_2. Started 4th day of experiment.

	Cortisone treated		Controls	
Days in 100% O_2	No. dead	No. sacrificed	No. dead	No. sacrificed
1	—	—	—	—
2	1	5	—	6
3	3	2	—	5
4	5	1	1	5
5	5	—	2	2
6	2	—	1	4

In the second experiment, essentially the same procedure was followed. A daily injection of 1 mg cortisone was given to each of 27 mice while the 26 controls received daily injections of comparable amounts of the diluent. All animals were placed in the chamber on the fourth day of the experiment. Commencing the second day of oxygen exposure, the chamber was opened just long enough to permit removal of the dead mice and of a number of the survivors which were sacrificed for histological study of their lungs. Sacrifices were made by cervical fracture. After resealing the lid, oxygen was admitted at a high rate of flow to purge the chamber of nitrogen and reestablish the 100% oxygen atmosphere.

The mortality rates (Table II) were similar to those of the first experiment, the cortisone treated animals proving to be less resistant to the toxic effects of the high oxygen atmosphere. The lungs of the control animals sacrificed on the third day of exposure to 100% oxygen were normal on both gross and microscopic examination (Fig. 1) while those of the cortisone treated animals sacrificed on the same day showed intense engorgement of the alveolar capillaries (Fig. 2). The same early findings were not observed in those sacrificed animals in the control group until the sixth day of exposure, by which time all of the cortisone treated animals had died (Fig. 3).

The lungs of the cortisone treated animals that died were dark red in color and grossly congested. Microscopic examination (Fig. 4) revealed intense engorgement of the alveolar capillaries. The alveoli contained an exudate with mononuclear cells and polymorphonuclear leucocytes and fibrin which, in many instances, had formed a hyaline membrane. There was some perivascular exudate. The same changes were present in the peribronchial areas. Essentially the same finding was noted in the lungs of the control animals that died. Thus, the final picture was the same in both groups of animals except that all of the pathological alterations occurred significantly earlier in the cortisone treated animals.

Discussion. The demonstration of the effectiveness of cortisone in controlling status asthmaticus has resulted in wide use in the treatment of bronchial asthma. The fact that many asthmatic patients also have concurrent infectious disease of the lungs, as well as the fact that a patient treated with cortisone for non-pulmonary conditions may develop an independent pulmonary infection, prompts a consideration of the effects of cortisone on such pulmonary diseases.

Mice treated with cortisone have been shown to have increased susceptibility to pneumococcal and influenza A infections(7) and similar results have been demonstrated in streptococcal infections in rabbits(8,9). The enhancement of the lethal effects of an acute pneumonitis of non-infectious origin as shown by the above report emphasizes that cortisone depresses resistance to pulmonary inflammation. These data suggest that cortisone is contraindicated in the treatment of patients with pulmonary infections and that patients who are susceptible to acute pulmonary infection should be kept under careful observation when given this drug for other diseases.

While exposure to 100% oxygen at atmospheric pressure is not deleterious to humans, the finding that cortisone increases the susceptibility of lower animals (Rodentia) to its toxic effects suggests that consideration be given to the possibility that high concentrations of oxygen be administered cautiously to patients receiving cortisone.

Summary. 1. Cortisone was shown to increase the susceptibility of mice to acute pneumonitis induced by high oxygen atmosphere. 2. Pathologic changes appeared earlier in the lungs of the cortisone treated animals and the mortality rate was accelerated. 3. The terminal histologic findings in the lungs of the cortisone treated animals and the controls were qualitatively similar. 4. It is suggested that cortisone is contraindicated in the treatment of acute pulmonary infections. 5. Attention is called to the possibility that high oxygen atmospheres may be deleterious to patients receiving cortisone.

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Effects of ACTH, Cortisone and Adrenal Cortical Extract on Carbohydrate Metabolism of Hypophysectomized Dogs.*† (19618)

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The Addisonian patient and the adrenalectomized animal exhibit severe disturbances in carbohydrate metabolism(1,2), which can be rectified to a great extent by administration of cortisone(1,3). Massive doses of either ACTH or cortisone in the normal animal can produce a diabetes-like condition(4). The hypophysectomized animal exhibiting severe adrenal cortical atrophy also shows evidence of derangements in its carbohydrate metabolism; this animal manifests hypersensitivity to the hypoglycemic action of insulin(5-7), a secondary hypoglycemia in the glucose tolerance test(7), and a diminished response to the hyperglycemic action of adrenaline(8,9). This paper is concerned with the effects of 1) adrenal cortical extract (ACE), 2) cortisone, and 3) ACTH upon these defects of carbohydrate metabolism in the hypophysectomized dog. In this way the importance of the adrenal cortical atrophy in the disturbed carbohydrate metabolism of the hypophysectomized dog may be further clarified.

Methods. The investigations were performed on 11 trained, unanesthetized male and female hypophysectomized dogs. The technic of hypophysectomy(8), the after-care and the feeding(7) of these animals have been described elsewhere.§ As a prerequisite for inclusion in this study, the dogs had to exhibit a high degree of insulin hypersensitivity and marked resistance to the hyperglycemic action of adrenaline. All experiments were done in the postabsorptive state. Insulin sensitivity was determined by the hypoglycemic response to 0.025 unit/kg insulin (Lilly),§ given intravenously (designated hereafter as the test dose); blood sugar changes were followed for 4 hours. Glucose tolerance was determined by the blood sugar response to a 10-minute intravenous infusion of glucose, 0.075 g/kg/min.; blood sugar changes were again followed for 4 hours. The response to the hyperglycemic action of adrenaline was tested by a 5-minute intravenous infusion of adrenaline hydrochloride, 0.0035 mg/kg/min. The ACE used was kindly supplied by Dr. E. C. Kendall; 1 cc of this extract was prepared from 150 g adrenal gland. The usual dose of ACE was 25 cc; the route and time of administration were varied, but the optimal effects with this dose were achieved when given as follows: 5 cc intramuscularly 17 to 18 hours before the experiment, 10 cc intramuscularly one hour prior to the injection of insulin, and again 10 cc intravenously immediately before insulin. Cortisone acetate (Cortone acetate, Merck) was given intramuscularly twice daily in doses ranging from 20 to 50 mg/day for periods of 65 to 86 days. Most of the ACTH preparations used were prepared and kindly supplied by Dr. R. W. Bates of E. R. Squibb and Sons. The following Squibb preparations were used: Lot No. P232Ac (20 IU/mg), C15Pr (3 IU/mg), P273Ac (10 IU/mg), and D13Pr (4 IU/mg). The daily dosage of these preparations ranged from 3 to 10 IU/kg and was administered intramuscularly in 4 divided doses (9 A.M., 1 P.M., 5 P.M., and 9 P.M.) for periods of 23 to 125 days. In addition, a few dogs received Armour ACTH§ preparations in daily dosages of 1 to 3.5 mg/kg for periods of 49 to 75 days prior to the adminis-

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‡ Dazian Foundation Fellow, 1950-51. Present address: Mount Sinai Hospital, New York City.

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TABLE I. Effect of Adrenal Cortical Extract (ACE) on the Insulin Response of Hypophysectomized Dog H-5.

	Time, min	Control	ACE-Rx*
		Blood sugar in mg %	
(A) First 4-hr test	0	71	78
	Insulin (.025 μ/kg) intrav. at 0 time		
	10	58	62
	20	49	45
	30	46	42
	40	39	42
	50	38	41
	60	38	47
	75	35	51
	90	36	56
	120	39	67
	150	43	72
	180	43	77
	210	48	78
	240	43	79
(B) Second 4-hr test	0 (240)	43	79
	Insulin (.025 μ/kg) intrav. at 0 (240) time		
	10 (250)	46	73
	20 (260)	42	61
	30 (270)	38	57
	40 (280)	36	54
	50 (290)	34	51
	60 (300)	33	53
	75 (315)	31	59
	90 (330)	30	61
	120 (360)	33	71
	150 (390)	37	75
	180 (420)	39	78
	210 (450)	45	80
	240 (480)	47	82

* Dose of ACE (Kendall): 25 cc i.m. (see text for schedule).

tration of any Squibb ACTH preparation. The Armour ACTH preparations used were lot No. 58-9-50 and 212-103 (both having potencies of 1 mg = 2 La-I-A Armour Standard).

Results. I. *Experiments with ACE.* Sixteen insulin sensitivity tests and 12 adrenaline experiments were performed on hypophysectomized dogs 233 to 456 days post-hypophysectomy. ACE, in the above dosage, exerted a marked anti-insulin effect in all the experiments, *viz.*, it prevented the fall of the blood sugar to the low level seen in the untreated hypophysectomized state and accelerated the recovery of the blood sugar to the pre-insulin injection levels. This is illustrated in Part A of Table I which records the insulin response of hypophysectomized dog H-5. Since the maximal effect of the 2 injections of ACE (given within the 1-hour period prior to insulin) seemed to occur 4 to 6 hours later, an 8-hour insulin sensitivity test was devised in which a second injection of insulin was given at the conclusion of the first 4-hour period and blood sugar changes again followed for 4 hours. Thus, 2 successive 4-hour insulin sensitivity tests were conducted after the ACE injections. Table I records such a typical 8-hour test in which Part A represents the first 4-hour test period and Part B, the second 4-hour test period. As can be seen, the maximal effect occurred in the second 4-hour test period (Part B, Table I). In no case did ACE, in the dosages given, completely abolish the insulin hypersensitivity of the hypophysectomized dog. At the same time, such signs as ataxia, inability to stand and photophobia, present in the untreated hypophysectomized animal after insulin, did not appear after ACE administration. In the above experiment, the effect of ACE on the post-absorptive blood sugar was slight, but in other experiments, ACE was seen to elevate the postabsorptive blood sugar to a greater degree. The glycemic response to adrenaline in these animals showed only a small increase after ACE administration, and no animal had a response comparable to that of a normal dog.

II. *Experiments with cortisone.* Twenty experiments, testing the insulin sensitivity, glucose tolerance and adrenaline resistance,

TABLE II. Insulin Sensitivity Tests in Hypophysectomized Dog H-3 before and during Cortisone Administration.

Time, min	Days of cortisone-Rx 0	15*	40†
	Blood sugar in mg %		
0	64	70	72
Insulin (.025 μ/kg) intrav. at 0 time			
10	55	70	71
20	51	64	67
30	43	61	63
40	39	60	70
50	37	61	72
60	38	63	74
75	40	69	75
90	44	71	78
120	45	73	80
150	56		
180	56		
210	58		
240	58		

* Dose of cortisone: 20 mg/day i.m.

† " " " increased to 50 mg/day 11 days prior to exp.

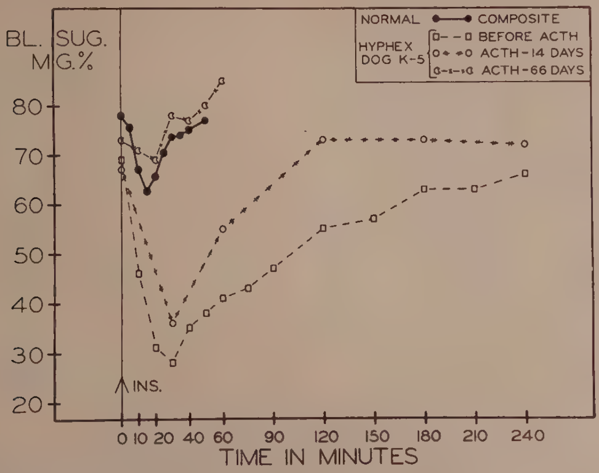

FIG. 1. Blood sugar curve produced by insulin (.025 μ/kg i.v.) in normal dogs compared with those of hypophysectomized (hyphex) Dog K-5 before and during ACTH regimen. Armour ACTH #212-103 was given for 49 days and followed by Squibb ACTH #C15Pr for 17 days.

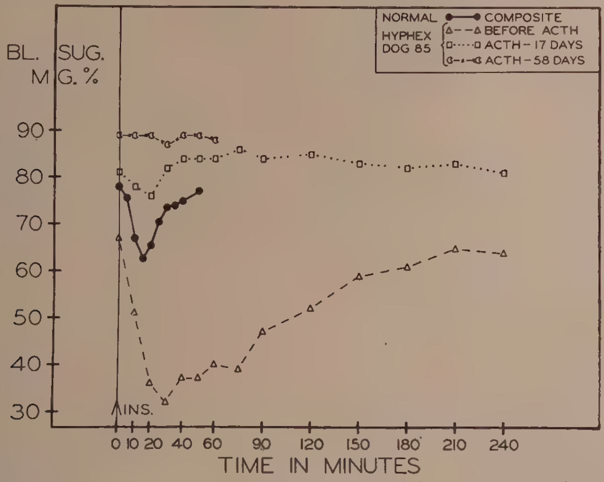

FIG. 2. Blood sugar curve produced by insulin (.025 μ/kg i.v.) in normal dogs compared with those of hypophysectomized (hyphex) Dog 85 before and during ACTH regimen. Only Squibb ACTH #C15Pr given for 58 days.

were done on a series of hypophysectomized animals 71 to 237 days post-hypophysectomy. The animals first were given 20 mg of cortisone per day, and their dosage was raised gradually to 50 mg/day. The changes in insulin sensitivity produced by cortisone in a representative hypophysectomized dog H-3 are recorded in Table II. As can be seen, 15 days of cortisone administration to dog H-3 (20 mg/day): 1) produced a rise in the post-absorptive blood sugar to normal, 2) diminished considerably the previously marked blood sugar fall in response to insulin, and 3) returned the blood sugar to the pre-injection levels in a short period of time. Thus, cortisone exerted a marked anti-insulin effect at this time. The dose of cortisone was increased and an experiment done on the 40th day revealed a further change in the blood sugar curve so that there was complete abolition of the insulin hypersensitivity of dog H-3. This response to the test dose was similar to that seen in normal animals. The other dogs used in this phase of the investigation exhibited similar results in that cortisone administration produced a marked anti-insulin effect; the degree of response varied directly with the amount and duration of the dosage. Concomitant with the abolition of the insulin hypersensitivity in these hypophysectomized dogs, cortisone changed the glucose tolerance so that the secondary hypoglycemia characteristic of the hypophysectomized dog was either significantly reduced or abolished. In addition, the glycemic response to adrenaline during cortisone administration approached or exceeded the normal range.

III. *Experiments with ACTH.* A total of 17 insulin sensitivity tests, 10 glucose tolerance tests, and 9 adrenaline tests were performed on a series of hypophysectomized dogs in order to assess the effect of ACTH on their carbohydrate metabolism. The ACTH regimens were begun 85 to 570 days post-hypophysectomy. In all the animals, continued administration of ACTH first diminished the exaggerated response to insulin, then elevated the post-absorptive blood sugar and abolished the insulin hypersensitivity thereby producing a normal response to insulin (Fig. 1). Prolonged administration of ACTH produced a resistance to the test dose of insulin inasmuch as no fall at all in the blood sugar was evident after the injection of insulin and usually elevated the postabsorptive blood sugar to above normal (Fig. 2). It is of interest to note that a potent Squibb preparation of ACTH was able to produce complete abolition of the insulin hypersensitivity in some animals as early as 8 days after the onset of the regimen. In other dogs this effect was seen only after longer periods of administration of the same preparation. Likewise, resistance to the test dose of insulin appeared at varying intervals after the ACTH regimen was begun; this was true when the same preparation was tested in different animals.

Concomitant with these effects, ACTH changed the glucose tolerance of these dogs in that there was always evident, at the end of the intravenous glucose infusion, an elevation in the peak of the blood sugar curve which was above that seen in the untreated hypophysectomized dog. Furthermore, the secondary hypoglycemia seen in the glucose tolerance of the untreated dog was completely eliminated. In some of the animals, no further change was evident but in a few dogs a tendency toward a diabetic-type of glucose tolerance was present in that the peak of the blood sugar curve at the end of the infusion was elevated far more and the slope of the declining blood sugar curve was shifted to the right (Fig. 3).

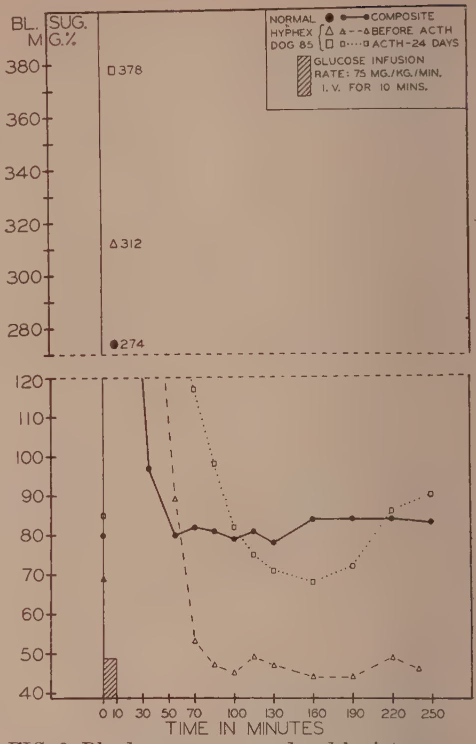

FIG. 3. Blood sugar curve produced by intravenous glucose (.75 g/kg) in normal dogs compared with those of hypophysectomized Dog 85 before and during ACTH regimen. Only Squibb ACTH #C15Pr given for 24 days.

Before ACTH administration, all the hypophysectomized dogs exhibited only slight rises in blood sugar (maximum increase: 14 to 20 mg %) after the intravenous administration of adrenaline. These results agree with previous work reported on the response to adrenaline infusion of untreated hypophysectomized dogs (8,9). During the ACTH regimen, the blood sugar rise in response to adrenaline increased (maximum rise: 65 to 94 mg %); this was greater than that seen in normal dogs—average rise: 49 mg %(8,9). In 2 dogs, the rises were within normal range (33 and 43 mg %, respectively). Only one dog had an exceptional rise of 36 mg % before ACTH, but during ACTH regimen this dog exhibited a rise of 88 mg %.

The weights of the 2 adrenal glands were within the normal range (1.5 to 3.0 g) in 4 ACTH-treated hypophysectomized dogs(9); of the remaining dogs in this series, 2 others thus far studied had adrenal weights which were not yet within the normal range.

Discussion. It is obvious from the above results that cortisone and the adrenal corticosteroids secreted under the influence of ACTH administration exert a potent influence in rectifying the defects in carbohydrate metabolism of the hypophysectomized dog. These hormones elevated the postabsorptive blood sugar and abolished the insulin hypersensitivity,¶ the secondary hypoglycemia following the intravenous administration of glucose and the adrenaline resistance of these dogs. ACE produced the same effects qualitatively but to a much lesser degree; this may be attributed to the smaller quantities of steroid present in the dosage schedule employed. However, in the experiments with cortisone and ACTH, effects of overdosage or oversecretion were observed, namely, 1) resistance to the test dose of insulin, 2) a tendency towards the production of a diabetic postabsorptive blood sugar level and glucose tolerance, and 3) a greater glycemic response to adrenaline than seen in normal dogs.

There is no doubt that the adrenocortical hormones can produce a significant reversal in the many abnormalities of carbohydrate metabolism outlined above. It is most probable, therefore, that the adrenal cortical atrophy of the hypophysectomized dog is of great importance in the production of these defects. The exact part played by the adrenal cortical atrophy in the disturbed carbohydrate metabolism of hypophysectomized animals and, consequently, the exact role of the adrenal cortex in the regulation of normal carbohydrate metabolism cannot be accurately evaluated from the results of the above experiments. This would have been possible only if physiological hormone levels had been obtained by the replacement therapy used in these experiments. In view of this, a comparison of the carbohydrate metabolism of the adrenalectomized dog with that of the hypophysectomized dog may further clarify the importance of the adrenocortical hormones in the regulation of carbohydrate metabolism.

Summary and conclusions. Prolonged administration of ACTH and cortisone diminished and eventually abolished the insulin hypersensitivity of the hypophysectomized dog. Concomitant with these effects, ACTH and cortisone also abolished the secondary hypoglycemia of the intravenous glucose tolerance test and produced a marked blood sugar rise in response to adrenaline. In some animals, however, there appeared some degree of insulin resistance together with a diabetic-type of glucose tolerance and an increased response above normal to adrenaline. It is concluded, therefore, that the adrenal cortical atrophy of the hypophysectomized dog exerts a significant part in the production of the insulin hypersensitivity of this animal. Although the adrenal cortical steroids are potent anti-insulin agents, their exact role as anti-insulin agents cannot be delineated in view of the evidence that abnormal amounts of adrenocortical steroids were present in the above studies.

¶ With regard to the abolition of the insulin hypersensitivity, the adrenocortical steroids may be aided, in part, by the glycogen-mobilizing action of adrenaline secreted by the adrenal medulla of the hypophysectomized dog. In the untreated hypophysectomized dog, the blood sugar-raising effect of intravenously administered adrenaline is minimal (8,9).

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Comparison of Insulin Hypersensitivity of Adrenalectomized and of Hypophysectomized Dogs.*† (19619)

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In a previous publication it was shown that the prolonged administration of ACTH or cortisone abolished the insulin hypersensitivity of the hypophysectomized dog(1). However, as an accompaniment of this effect, a tendency towards diabetes appeared which was strongly suggestive of either excessive adrenal steroid secretion or replacement, respectively, in these animals.

Since physiological hormone levels were not obtained in the above experiments, a comparison of the insulin hypersensitivity of the adrenalectomized animal with that of the hypophysectomized animal was made in order to determine to what extent the adrenal cortical atrophy of the hypophysectomized dog is responsible for its insulin hypersensitivity. A report of these studies is presented in this paper.

Methods. In this investigation 7 trained, unanesthetized, adrenalectomized‡ female dogs were used. All the animals were fed a mixed diet and maintained in good condition with minimal doses (1.5 to 2.5 mg daily) of desoxycorticosterone acetate (DCA) in oil (Schering)‡ administered intramuscularly. All experiments, unless otherwise stated, were done in the postabsorptive state, 17 to 18 hours after the last feeding. The insulin sensitivity of these dogs was determined 7 to 323 days postoperatively and tested with insulin (Lilly),‡ 0.025 u/kg, i.v., (referred to as the test dose), and 0.25 u/kg, i.v. In all the adrenalectomized dogs the sensitivity was tested with both doses of insulin while the animals were maintained on DCA. With the exception of one dog, all the others were then taken off maintenance DCA and tested repeatedly during a DCA-free period (lasting 8 to 14 days), until signs of adrenocortical insufficiency appeared. Some of these dogs were subjected several times to such DCA-free periods. Serum sodium concentration (measured on an internal standard flame photometer) was determined while each animal was on DCA and then was followed during the DCA-free period. No remedial measures were undertaken to alleviate the signs of severe adrenocortical insufficiency until the last insulin sensitivity experiment had been performed. Only then were the animals given adequate intravenous infusions of dextrose and saline several times daily, in addition to intravenous injections of adrenal cortical extract and intramuscular injections of large doses of cortisone as well as the maintenance dose of DCA. The insulin sensitivity of the hypophysectomized dogs used as a basis for comparison in this paper was compiled from a series of 38 tests

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TABLE I. Blood Sugar Changes Produced by Insulin (Test Dose) in Adrenalectomized (Adrex) Dog A-8 Compared with Normal and Hypophysectomized (Hyphex) Dogs.

		Adrex A-8			
		On	Off DCA		
	Normal*	DCA	6 days	9 days	Hyphex†
		Serum Na in mEq.			
		156	144	116	
Time, min	Blood sugar in mg %				
0	78	84	79	81	66
Insulin .025 u/kg given intrav. at 0 time					
10	67	77	71	68	53
20	66	75	71	65	38
30	74	75	77	73	36
40	75	80	78	73	35
50	77	81	75	73	36
60		83	76	79	37
75		81	78	78	38
90		79	78	76	42
120		85	77	76	47
150		83	74	73	51
180		84	73	76	54
210		83	75	73	56
240		79	74		58

* Values obtained from 37 experiments on 18 normal dogs.

† Values obtained from 38 experiments on 12 hypophysectomized dogs.

TABLE II. Blood Sugar Changes Produced by Insulin (Test Dose) in Adrenalectomized (Adrex) Dog A-2 Compared with Normal and Hypophysectomized (Hyphex) Dogs.

		Adrex A-2			
		On	Off DCA		
	Normal*	DCA	3 days	6 days	Hyphex†
		Serum Na in mEq.			
		141	—	127	
Time, min	Blood sugar in mg %				
0	78	69	65	70	66
Insulin .025 u/kg given intrav. at 0 time					
10	67	55	52	40	53
20	66	42	43	38	38
30	74	52	44	40	36
40	75	47	47	44	35
50	77	43	48	42	36
60		47	48	46	37
75		49	54	51	38
90		52	56	55	42
120			58	53	47
150			59	51	51
180			63	56	54
210			59	51	56
240			61	53	58

* Values obtained from 37 experiments on 18 normal dogs.

† Values obtained from 38 experiments on 12 hypophysectomized dogs.

performed on 12 animals and reported in other publications. This group of animals includes only those dogs which were found to be "completely" hypophysectomized upon histological examination of the pituitary region utilizing a serial section technic reported elsewhere(2).

Results. I. *Response to the test dose of insulin (0.025 u/kg, i.v.).* Table I records the experimental data obtained on dog A-8, which is representative of a group of 5 of the 7 adrenalectomized animals studied. Table II records the results of the insulin response of dog A-2, which is typical of those exhibited by the 2 remaining dogs in this series. As seen in Table I, dog A-8 exhibited an insulin response similar to that seen in normal dogs. This was true of the animal's response while on DCA maintenance, or even without DCA maintenance for a period of 9 days, although the animal developed signs of adrenal insufficiency, *viz.*, anorexia, weakness, apathy, and had a progressively declining serum sodium level. These results are in striking contrast to the marked insulin sensitivity of the hypophysectomized dogs recorded in Table I. The insulin sensitivity of dog A-8 and of the other 4 dogs in this group increased markedly only when their adrenal insufficiency became severe enough to cause the animals to vomit even force-fed food; thus, the insulin response obtained was that of animals fasted more than 40 hours.

Dog A-2 (Table II), on the other hand, showed an insulin response greater than that of normal dogs but less than that of hypophysectomized dogs. Here, too, the response obtained during a test period of 8 days without DCA maintenance showed little change from that obtained while the dog was on DCA maintenance. This was true, although the dog began to exhibit signs of adrenal insufficiency and had a lowering of the serum sodium level. Once again, the onset of vomiting, signifying a more severe state of insufficiency, introduced prolonged fasting (40 hours or more) into the experimental conditions, and the insulin response of the animal increased over that seen in all the experiments performed in the postabsorptive state (17 to 18 hours fasted).

Fig. 1 is a graphic presentation of the insulin responses to the test dose of normal and hypophysectomized dogs compared with the composite response obtained on the 7 ad-

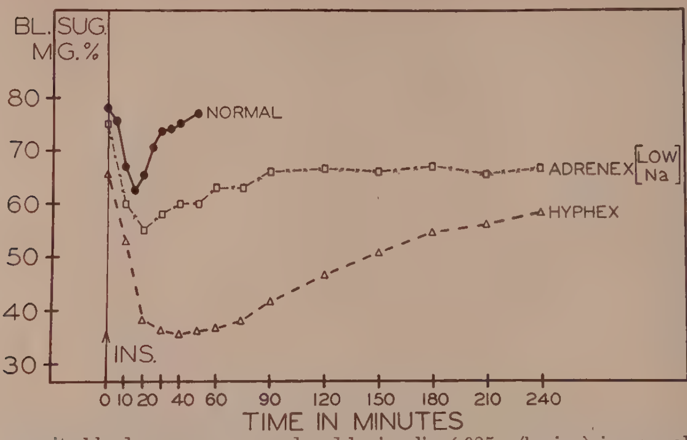

FIG. 1. Composite blood sugar curves produced by insulin (.025 u/kg i.v.) in normal, hypophysectomized (Hyphex) and adrenalectomized (Adrenex) dogs.

renalectomized dogs during DCA-free periods, when all 7 animals exhibited decreases in their serum sodium concentrations which were significantly below normal.

II. *Response to 10X test dose of insulin (0.25 u/kg, i.v.).* Since 5 of the 7 adrenalectomized dogs exhibited an insulin response to the test dose similar to that of normal dogs, the response to a larger dose of insulin (10X the test dose = 0.25 u/kg, i.v.) was examined.

TABLE III. Blood Sugar Changes Produced by Insulin (10 × Test Dose) in Normal Dogs and Those Adrenalectomized (Adrex) Dogs Not Sensitive to Test Dose of Insulin.

		Adrex dogs	
	Normal*	A-8‡	Comp.†‡
Time, min	Blood sugar in mg %		
0	78	70	74
Insulin .25 u/kg given intrav. at 0 time			
10	61	57	60
20	46	36	38
30	—	24	31
40	45	24	25
50	—	21§	29
60	52		32
75	—		36
90	66		48
120	76		54
150			55
180			62
210			61
240			64

* Values obtained from 17 exp. on 8 normal dogs.

† " " " experiments on 4 adrenalectomized dogs.

‡ Dose of DCA: 2-2.5 mg i.m. daily.

§ Convulsions; experiments discontinued.

Table III records the response to 10X the test dose of insulin of dog A-8, one of these 5 adrenalectomized dogs. Included, too, in Table III is the composite response of the remaining 4 dogs which were similar to A-8 in their response to the test dose of insulin. These are then contrasted with the response of normal dogs to 10X the test dose. As can be seen, all the adrenalectomized animals manifested a greater sensitivity than normal dogs. The hypophysectomized dog's response to the larger dose of insulin is not recorded, since the response to the test dose, 0.025 u/kg, is so marked and at times accompanied by convulsions that this consideration militates against the use of 10X the test dose.

Discussion. It is evident from the work presented that the adrenalectomized dog is more sensitive to insulin than the normal dog but less sensitive than the hypophysectomized dog. The experiments with 0.25 u/kg (10X the test dose) clearly demonstrate the difference in insulin response of normal and adrenalectomized dogs, whereas the experiments with the test dose (0.025 u/kg) reveal the differences obtained in the insulin response of normal, hypophysectomized and adrenalectomized dogs. Most of the adrenalectomized dogs had insulin responses with the test dose similar to those of normal dogs, whereas 2 reacted to insulin to a greater degree than do normal animals. However, all the adrenalectomized dogs were less sensitive to insulin than the hypophysectomized dogs, even when the former were without DCA maintenance for 8 to 14 days and showed evidence of being steroid-free by virtue of their low serum sodium level. The fact that the adrenalectomized animal in the pre-mortal state, lacking the ability to retain food and thereby fasted for more than 40 hours, has a marked increase in insulin sensitivity does not contribute to the evaluation of this particular problem since comparison of the insulin response is being made to the postabsorptive hypophysectomized animal.

These results are not in contradiction with the earlier studies of other investigators who demonstrated the insulin sensitivity of adrenalectomized animals. Rather, these experiments can be considered as an extension of those studies in order to evaluate more accurately the degree of sensitivity of the adrenalectomized dog, utilizing a more quantitative approach to the problem. Grattan, Jensen, and Ingle(3) obtained convulsions in adrenalectomized mice with doses of insulin (0.4 u/kg) which were much larger than our test dose; no smaller dose of insulin was used in their study. This is true, too, of other workers (Zucker and Berg(4)), who studied the insulin sensitivity of the adrenalectomized dog but, again, used doses which were larger than the test dose employed in the current study.

The insulin sensitivity of the adrenalectomized dog is not entirely due to the lack of the adrenal cortical hormones, since it lacks adrenaline, too, which exerts an anti-insulin action although this is not as potent as that of the corticosteroids. The hypophysectomized dog has a diminished secretion of adrenocortical steroids due to the adrenal cortical atrophy, and furthermore, the adrenaline from its adrenal medulla, if secreted, exerts little, if any, blood sugar raising action(2,7). Undoubtedly, the adrenal cortical steroids are effective anti-insulin agents in the hypophysectomized dog as seen from the previously reported work on the effects of ACTH and cortisone on the insulin hypersensitivity of the hypophysectomized dog(1). Nevertheless, the adrenal cortical atrophy of the hypophysectomized dog cannot wholly explain its marked hypersensitivity. It follows, therefore, that in the production of the insulin hypersensitivity of the hypophysectomized animal, the absence of anti-insulin agents of pituitary origin must be implicated. In view of previous work demonstrating the anti-insulin and diabetogenic effects of growth hormone (Armour) in the hypophysectomized dog(5), it would appear that growth hormone is the absent anterior pituitary anti-insulin agent in these animals. In more recent work, however, a growth hormone preparation without any diabetogenic effect seems to have been isolated(6). Whether the anterior pituitary hormone exerting the marked anti-insulin effect in the hypophysectomized dog is the growth-promoting factor itself or another anterior pituitary factor intimately associated with it is of no consequence to this discussion. The fact remains that there is an anterior pituitary factor (in addition to ACTH) that is an effective anti-insulin agent and the lack of this agent is important in the production of the marked insulin hypersensitivity of the hypophysectomized dog.§

Summary and conclusions. The adrenalectomized dog is more sensitive to insulin than the normal dog but less sensitive than the hypophysectomized dog. This is true so long as the adrenalectomized dog, whether with or without DCA maintenance, is still well-nourished and tested only in the post-absorptive state. It is concluded that the adrenal cortical atrophy is an important factor in the production of the insulin hypersensitivity of the hypophysectomized dog. However, absence of an anterior pituitary factor (other than ACTH) is also of great importance in the production of this metabolic abnormality.

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§ It is possible that an over-secretion of this anterior pituitary factor (growth hormone?) accompanies the over-secretion of ACTH in the adrenalectomized animal and exerts an anti-insulin action. However, the glucose tolerance of the adrenalectomized dog does not reveal any evidence for the over-secretion of such a factor.

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Effects of C_{17}-Hydroxy-Steroids on Urinary 17-Ketosteroids in the Rat.* (19620)

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Because all the urinary 17-ketosteroids (17-KS) of females and some part of those excreted by males originate in the adrenal cortex, their determination is one of the means of evaluating adrenocortical function. The association of 17-KS content with androgenic properties, the presence of adrenosterone in the adrenal cortex and the masculinization elicited by some forms of adrenal cortical hyperfunction have supported the concept of a specific androgenic secretion of the adrenal cortex. However, recent evidence(1-6) indicates that 17-KS are formed and excreted during metabolism of corticosteroids, particularly of those hydroxylated at C 17. Relevant studies in human subjects are scanty. Those here reported were undertaken in rats, in which relatively large doses of potential 17-KS precursors could be readily administered.

Methods. Female albino rats (Sprague-Dawley) of 150-250 g body weight were separated into 7 groups, 5 experimental and 2 control; each group contained at least 4 rats. The animals were placed separately in metabolism cages for a period of 13 days; in order to accustom the animals to this environment, 4 days elapsed before real or simulated treatment was begun; during the treatment period, 10 mg of each steroid tested was given by stomach tube daily for 5 days; 4 days were allowed for post-treatment observation. Drinking water was substituted for with 1% NaCl in order to obtain a diuresis; commercial fox chow was given *ad libitum*. A few drops of Merthiolate solution were placed in the collection bottles; the urine was collected and measured daily. The steroids administered were as follows: 11-desoxycorticosterone acetate (DCA); 17-hydroxy-11-desoxycorticosterone acetate (compound S); progesterone; 17-hydroxyprogesterone and Δ^4-pregnene-17 α, 20, 21-triol-3-one diacetate (pregnenetrio-

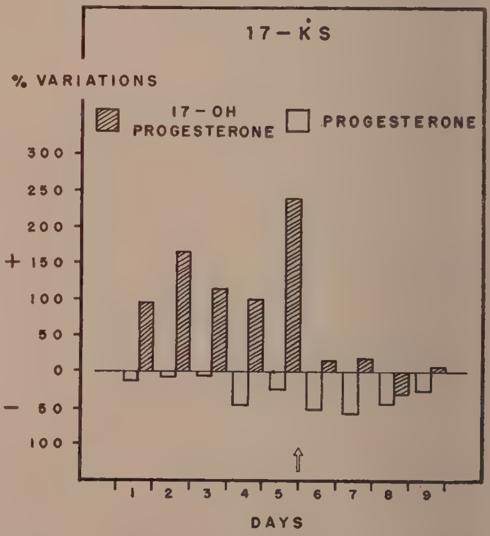

FIG. 1. % variations from control mean of urinary 17-ketosteroids following administration of progesterone and 17-hydroxyprogesterone. Arrow indicates end of treatment.

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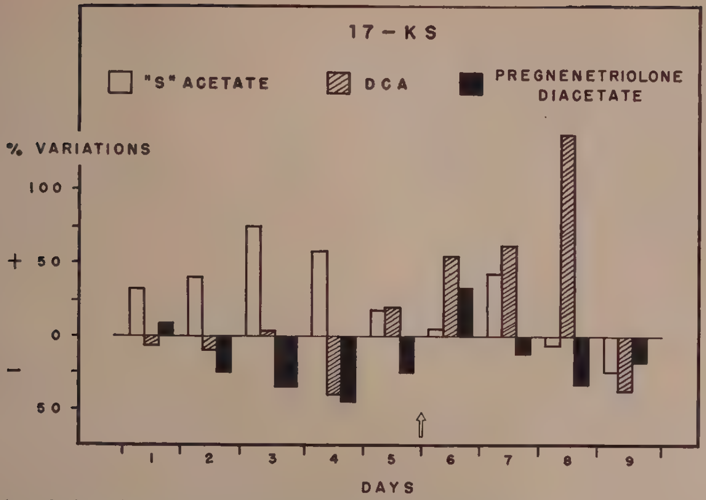

FIG. 2. % variations from control mean of urinary 17-ketosteroids following administration of compound S acetate, desoxycorticosterone acetate and pregnenetriolone diacetate. Arrow indicates the end of treatment.

lone). The steroids were given in water suspensions containing 10 mg per cc; one group of controls was gavaged daily with 1 cc of saline; the other control group was untreated. The method of 17-KS determination was that of Drekter *et al.*(7). It was found advantageous because it can be rapidly completed, requires small amounts of urine and eliminates chromogens which were particularly troublesome in rat urines with other methods. The values obtained are expressed in terms of a dehydroisoandrosterone standard.

Results. In the control groups, administration of saline by gavage had little effect on 17-KS outputs; individually some animals which resisted the procedure showed initially higher outputs which later stabilized at lower levels; similarly, 17-KS outputs of some animals were about 30% higher during the first day or two than they were after they had become accustomed to the metabolism cages.

From 55 determinations in normal females, obtained after achievement of stable levels in this and other experiments, the 17-KS output of the rat was found to average 198 μg per 24 hours (standard error of the mean ±7.9 μg). Individuals differed fairly widely in their outputs; these were relatively constant in undisturbed animals.

Results from the experimental groups are summarized in Fig. 1 and 2; changes caused by treatment are expressed as percentage variation from the means of the last 3 days of the control periods. DCA and progesterone, which are devoid of a C-17 hydroxyl, do not increase 17-KS output; on the contrary both seemed to decrease slightly the output of 17-KS during treatment. That this decrease may be real in the case of DCA is suggested by the immediate rebound above control level observed during the post-treatment period. The 17-hydroxylated steroids, compound S and, to a greater extent, 17-hydroxyprogesterone increase 17-KS outputs; on the other hand, pregnenetriolone seemed to depress output of endogenous 17-KS without itself being excreted in this form. The significance of the increased outputs is established by the abrupt change in output observed when treatment was discontinued.

Discussion. The 17-KS outputs of normal female rats here reported agree with those of Kowaleski *et al.*(8); their mean of 190 μg per rat per day was obtained by Warren's modification of Callow's method(9). As might be expected, the values are greater than the mean of 30 μg found by Danford and Danford(10) who used the method of Engstrom and Mason(11) and separation of the ketonic fraction with Girard reagent.

In vitro, periodic acid oxidation of the 3 17-hydroxylated compounds tested in this study can yield the respective 17-ketosteroids (12,13); however, in the case of pregnenetriolone, the initial product of molar oxidation is a 17-hydroxyaldehyde(13). Similarly, *in vivo* in rats, 17-KS appear in urine as metabolic products of compound S and 17-hydroxyprogesterone, but not of pregnenetriolone. The marked effectiveness of 17-hydroxyprogesterone in augmenting urinary 17-KS output is of interest in view of the androgenic property of this steroid in castrate rats(14); it may be that its metabolism results in formation of biologically active 17-KS.

The data obtained from rats accord with observations in some human beings; in these, compound S(1,5) and 17-hydroxyprogesterone (1) increase urinary 17-KS while DCA(15) and progesterone(16) do not. Data are available from only 2 patients given pregnenetriolone(1); in one, the compound had no effect and in the other, the output of 17-KS seemed to double. The observations in rats suggest that this change may have been an artefact.

Technically, the procedure here described has definite advantages in the evaluation of 17-KS precursors. As noted above, it permits the administration of small amounts of steroids with observations in a sufficient number of animals; especially, its advantage lies in its relative independence of endogenous adrenocortical metabolism; thus, in human beings, cortisone has been found to have disparate effects of urinary 17-KS(17), apparently because in smaller dosage it suppresses endogenous adrenocortical function to a greater degree than it contributes to 17-KS output.

In brief, these observations in rats support the view that the adrenal precursors of urinary 17-KS are 17-hydroxylated compounds. Current unpublished observations show that compound F, which seems to constitute the bulk of adrenocortical hormone is a 17-KS precursor; in view of this and of the androgenic properties which seem to be associated with the metabolic products of 17-hydroxyprogesterone, it may be that the masculinizing effects of some forms of adrenocortical hyperactivity should be attributed to the secondary products of hypersecretion rather than to a primary secretion of specific androgens. Indeed, the evidence for androgenic secretion by the adrenal cortex is not substantial. It is based in large part on the presence of adrenosterone in extracts; this is considered by some to be artefactual(3).

Summary. A method is described for detecting precursors of urinary 17-KS in normal female rats. Using it, 2 pairs of compounds which differed in hydroxylation at C-17 were tested and those 2 possessing this grouping were found to be eliminated in part as urinary 17-KS. One 17-hydroxylated steroid, pregnenetriolone did not act as a 17-KS precursor. These observations bring into question the assumed secretion of adrenal androgens, since they are consistent with the view that such might arise from the degradation of non-androgenic hormones.

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Separate Identities of the Donath-Landsteiner Hemolysin (PCH Antibody) and Treponemal Immobilizing Antibody.* (19621)

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Paroxysmal cold hemoglobinuria (PCH) due to the presence of an abnormal serum hemolysin is recognized as a manifestation of syphilis, for nearly all patients with positive Donath-Landsteiner reactions have historical, clinical or serological evidence of syphilitic infection(1-3). The hemolysin is distinct from the Wassermann reagin(4,5), and surveys of non-hemoglobinuric patients with syphilis have detected only a few with positive Donath-Landsteiner reactions(6-8).

Since demonstration of the PCH hemolysin sometimes requires more complement than was used in the tests employed for these surveys (9), it was felt that a more sensitive test might reveal that many patients with syphilis have a detectable, but clinically inactive, hemolysin. Furthermore, because both the PCH antibody (10) and the treponemal immobilizing (TPI) antibody(11,12) are heat stable globulins that require complement for their activities, and because TPI antibody is a specific indicator of syphilitic infection, the relationship of these antibodies was investigated in an effort to clarify further the mechanism of production of syphilitic cold hemoglobinuria.

Materials and methods. Sera. For survey purposes, sera were obtained from hospitalized and clinic patients with strongly positive Wassermann and Kline reactions. The sera used as controls in the absorption experiment were obtained from patients who were not receiving penicillin or other antibiotics. The case histories of the 2 patients with paroxysmal cold hemoglobinuria have been summarized elsewhere(8). *Antibody tests.* The methods used for the following procedures were those previously described(8): 1) hemolysin titration, 2) indirect Coombs tests (a) and (b). The technic of Nelson and Diesendruck(13) was employed for the treponemal immobilization test.† *Absorption of sera.* Sera from the 2 patients with positive Donath-Landsteiner reactions and from 2 other patients with syphilis (controls), but with negative hemolysin reactions, were treated as follows:

Three ml of serum, 0.75 ml of freshly reconstituted lyophilized guinea pig serum, and 1.5 ml of fresh, thrice washed packed group O erythrocytes were added to a tube. This mixture was chilled in an ice bath for an hour, centrifuged in the cold, and the supernatant removed for testing. The erythrocytes used for absorption were washed 3 times with cold saline and tested for agglutination by antiglobulin serum in the indirect Coombs test (a). For each serum, 3 specimens, identified

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† Kindly performed by Mr. Murray Seldeen of the Department of Bacteriology, Johns Hopkins School of Hygiene and Public Health.

TABLE I. PCH Antibody Titers in Sera before and after Absorption.

Case	Specimen	Test	Initial dilution of serum Undiluted	2	4	8	16	32	64
1	#2	H*	3+	3+	3+	3+	2+	+	0
	Unadsorbed	A†	3+	3+	3+	2+	2+	2+	+
	#3	H	+	+	0	0	0	0	0
	Adsorbed	A	3+	2+	2+	0	0	0	0
2	#2	H	3+	2+	+	0	0	0	0
	Unadsorbed	A	2+	3+	2+	+	0	0	0
	#3	H	0	0	0	0	0	0	0
	Adsorbed	A	0	0	0	0	0	0	0

* Hemolysis in Donath-Landsteiner reaction.
† Agglutination in indirect Coombs test (b).

TABLE II. Effect of Absorption of PCH Antibody on Treponemal Immobilization Capacity of Syphilitic Sera.

	Specimen No.*	% Treponema motile With inactive C′	With active C′	Result	Residual complement
Case 1	1	80	0	+	4+
	2	80			
	3	72			
2	1	76			
	2	92			
	3	88			
Control 1	1	100			
	2	88			
	3	88			
2	1	84			
	2	84			
	3	88			

* Specimen No. 2 was absorbed (see text).

by number and complement content, were frozen and submitted for the TPI test. Specimen No. 1 was 1.25 ml of untreated serum; No. 2 was 1.25 ml of the absorbed serum and complement mixture, representing 1 ml of serum and .25 ml of reconstituted lyophilized guinea pig serum; No. 3 consisted of 1.0 ml of serum plus 0.25 ml of guinea pig serum.

Results. Survey. Sera from 33 patients with serological evidence of syphilis were titrated for hemolysin and indirect PCH antibody. No additional individuals with positive Donath-Landsteiner reactions were found.

Absorption. Only those erythrocytes used to absorb sera from patients with hemoglobinuria were agglutinated by antiglobulin serum. The effect of absorption on PCH antibody is shown in Table I.

Table II indicates that absorption of patient and control sera did not alter the treponemal immobilizing capacity of the sera. Thus, PCH antibody may be removed from sera without removing TPI antibody. The reverse experiment could not be performed because absorption of TPI antibody is not yet feasible (14).

Discussion. Paroxysmal cold hemoglobinuria in syphilitic patients is associated with a positive Donath-Landsteiner reaction. Conversely, patients with positive Donath-Landsteiner tests nearly always, if not always, have syphilis. Although syphilitic infection is responsible for the hemoglobinuria, very few patients with syphilis develop an autohemolysin. Cold hemoglobinuria in non-syphilitics can be attributed to the presence of potent cold hemagglutinins (3,15).

The low incidence of positive tests for PCH antibody as an indication of syphilitic infection (even utilizing a more sensitive test) is in contrast to the high incidence of positive Wassermann and treponemal immobilization tests. Both PCH(5) and TPI(11) antibodies have been shown to be distinct from the Wassermann reagin. Although both are globulins, the PCH antibody is a *gamma*-globulin(10) while the spirocheticidal TPI antibody appears to be associated with the *beta*-globulins (12). The present study has demonstrated that reduction or removal of PCH antibodies by absorption fails to alter the qualitative TPI reaction. Quantitative TPI titrations were not feasible(14), and it is possible that TPI antibody might have been removed without changing the outcome of a qualitative test. This is considered to be unlikely. The absorption results support the serum fractionation studies in indicating that the 2 types of antibody are not related, although final proof must await the development of a method for reciprocal absorption of the TPI antibodies. TPI antibodies appear as a specific response to infection with *Treponema pallidum*(11). The mechanism of development and the low occurrence of the PCH hemolysin in patients with syphilis remain unexplained.

Summary. A more sensitive Donath-Landsteiner test did not detect the hemolysin of paroxysmal cold hemoglobinuria in sera of patients with syphilis more often than tests used in other surveys. Although related to syphilitic infection, PCH antibody was shown to differ from treponemal immobilizing antibody, the specific antibody of this infection. The mechanism of production of PCH antibody remains unexplained.

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Inhibitory Effect of Antibiotics on Virus of Rous Sarcoma. (19622)

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Rous(1) described a sarcoma of chickens which was apparently caused by a filterable virus. This tumor is a spindle-shaped growth which is both infiltrative and destructive. The tumor can be transmitted by cell-free filtrates, by desiccated tumor tissue and by glycerine extracts. The tumor can also be transmitted by the blood, ascitic fluid and tumor-free organs of the tumor bearing animal providing the tumor is of considerable size. The more recent work of Porter and Thompson(2) and others on electron microscope examination of the tissue of the Rous sarcoma shows the presence of virus-like particles which are believed to be the etiological agent of this tumor.

This report deals with the effect of certain antibiotics on the virus of the Rous sarcoma of chickens. Penicillin, streptomycin, bacitracin, terramycin, chloramphenicol, aureomycin, neomycin and antibiotic PA96(Pfizer) were tested. Various concentrations of each

TABLE I. Effect of Antibiotics on Virus of Chicken Sarcoma *In vitro* at 10°C for 24 Hr.

	Min effective conc. for inhibition of virus	
Antibiotic	Tumor-cell suspension, mg/ml	Cell-free extract, mg/ml
Terramycin	166	40
Aureomycin	50	15
Neomycin	60	30
PA96	75	40
Penicillin	No effect up to	10000 μ/ml
Chloramphenicol		300 mg/ml
Bacitracin		10000 μ/ml
Streptomycin or dihydrostreptomycin		300 mg/ml

antibiotic were mixed with suspensions of the tumor cells and cell-free extracts of the tumor tissue and allowed to remain at ice box temperature (10°C) for 18 to 24 hours. The mixtures were then inoculated into susceptible, 3-week-old chickens. Control animals were inoculated with tumor tissue or tissue extract kept at ice box temperature but not treated with any antibiotic.

Terramycin, in a concentration of 166 mg per ml of tumor cell suspension inhibited whole tumor cells *in vitro* with complete loss of tumor producing activity. At a concentration of 40 mg per ml of cell-free extract the activity of the virus was destroyed by terramycin.

Aureomycin, in a concentration of 50 mg per ml of tumor cell suspension inhibited the tumor producing activity of whole tumor cells. At a concentration of 15 mg per ml of cell-free extract the activity of the virus was lost. Antibiotic PA96 was similar to aureomycin in its effect on the sarcoma virus. Experiments with neomycin indicate that this antibiotic also inhibits the activity of Rous sarcoma virus. Penicillin, streptomycin, bacitracin and chloramphenicol had no inhibitory effect on the virus in the concentrations tested. All animals inoculated with mixtures of each of these antibiotics and the virus developed tumors as rapidly and extensively as did the control animals.

For each antibiotic a total of 200 chickens were used in each experiment and repeat tests confirmed the original findings. The attached table summarizes the results of the effect of the antibiotics tested on the tumor-cell suspension and the cell free extract.

In another series of tests, animals were first infected with whole tumor cells or cell-free extract and then treated with terramycin and aureomycin. There was no apparent effect on the formation and development of tumors or the course of the disease in the chickens.

It was observed that with low concentrations of aureomycin, terramycin and bacitracin there was a stimulating effect on the development of tumors and the resulting growths were larger in size. The possible reasons for the ineffectiveness of treatment with the antibiotics which inhibit the virus *in vitro* may be due to the inability of the drugs to penetrate the intact cell where the virus is concentrated or that, once a tumor has developed, continued growth of the tumor depends upon proliferation of abnormal cells without virus stimulation.

Summary. 1. These experiments describe a method of testing the activity of chemotherapeutic drugs against whole tumor cells and against the cell-free agent of Rous chicken sarcoma. 2. Terramycin, aureomycin, neomycin and antibiotic PA96 (Pfizer) in certain concentrations were capable of preventing the formation of tumors of Rous sarcoma when the virus was first exposed to these antibiotics prior to inoculation into susceptible young chickens.

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Effects of Prolonged High Dosage with Ascorbic Acid.* (19623)

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In recent years relatively large doses of ascorbic acid have been prescribed in the treatment of a variety of diseases including hypertension and allergic conditions. It has been generally assumed that daily doses of one gram are innocuous although there have been reports that vagotonic symptoms occur in children and that animals receiving large quantities of ascorbic acid may lose weight(1).

Wilson and Lubschez(2) have reported preliminary data suggesting that possibly the ingestion of 500 to 1000 mg ascorbic acid per day for a long period might not be innocuous. These investigators found a paradoxical decrease in the ascorbic acid of the white blood cells after a time on high dosage of the vitamin and suggest that their results might arise from the organism becoming too efficient in the disposal of ascorbic acid.

Accordingly, it was felt desirable to investigate further the effect of prolonged high dosage with ascorbic acid. One woman and 3 men were each given 1000 mg/day for 3 months. Measurements were made at appropriate intervals of the ascorbic acid concentration in the serum, the concentration in the white blood cells plus platelets, the ascorbic acid tolerance curve, and the urinary output of the vitamin.

Methods and procedure. All analyses were performed at least in duplicate. The white blood cells plus platelets from 0.1 ml of finger blood were analyzed as previously described (3). The serum ascorbic acid was measured using 0.01 ml samples. The original procedure(4) was altered as follows: (a) charcoal was omitted from the precipitating acid; (b) the dinitrophenyl hydrazine reagent was prepared as follows: to a 2.2% dinitrophenyl hydrazine solution in 10 N H_2SO_4 is added 5 volumes of 5% thiourea solution and 5 volume % of 0.6% $CuSO_4 \cdot 5H_2O$ solution. The reagent is satisfactory for use at least a week if held at 4°C. It is prepared from the more stable stock solutions; and (3) the incubation time was increased from 3 to 4 hours at 38°C. The copper, which is easier to use, substitutes for charcoal in converting ascorbic acid to dehydroascorbic acid. This change gives results which average 0.1 mg % higher than with charcoal, a difference of no consequence in the present study. The urine was preserved with 0.1 N acetic acid and analyzed in the same manner as serum except the use of charcoal was retained as it has been found necessary to remove the urinary pigments. A single urine sample analyzed with dichlorophenol indophenol gave a value of 7% less than with dinitrophenyl hydrazine. This may indicate some conversion to dehydroascorbic acid prior to analysis, and suggests an advantage in the use of dinitrophenyl hydrazine which measures both ascorbic and dehydroascorbic acids. The 1000 mg daily supplement of ascorbic acid was administered in 3 divided doses (with meals). The 100 mg pills used were analyzed and found to be within 2% of the stated value.

Results and conclusion. The data for the 4 subjects were so concordant, they have been averaged together and treated as replicates. There is clearly no progressive change in either the serum or white cell level, tolerance curve, or urinary excretion (Table I). The averages obscure the fact that the individual tolerance curves differed a little in shape. However, the individual character of the curves remained essentially unchanged throughout. There seems, therefore, to be no reason to believe that the prolonged high dosage of ascorbic acid had any qualitative or quantitative effect on the manner of disposal of excess acid by the body. Furthermore, in these four

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TABLE I. The Ascorbic Acid of Urine, White Cells, and Serum of 4 Subjects Receiving 1000 mg Ascorbic Acid per Day.

Day of exp.	Urine, mg/day	White cells,* mg %	Serum after 400 mg test dose (mg %) 0	1 hr	3 hr	5 hr	7 hr
0		27 ± 2†	1.22 ± .09	1.78 ± .13	1.97 ± .11	1.80 ± .06	1.63 ± .04
5	817‡ ± 35†	28 ± 3	1.81 ± .09	2.45 ± .09	2.39 ± .08	2.14 ± .11	1.94 ± .04
21	804 ± 25	30 ± 1	1.79 ± .06	2.28 ± .11	2.35 ± .05	2.19 ± .11	2.03 ± .04
39	714§ ± 35	28 ± 1	1.82 ± .13				
55		28 ± 2	1.54 ± .08				
67		31 ± 1	1.93 ± .15	2.45 ± .17	2.48 ± .20	2.19 ± .24	2.09 ± .26
98	822 ± 47	28 ± 1	1.64 ± .10	2.24 ± .99	2.14 ± .08	1.83 ± .13	1.76 ± .06

* White blood cells + platelets. † ± Stand. error. ‡ 3rd day of exp. § 43rd day of exp.

persons, at least, no harmful effects whatever were observed during the 3 months with 1000 mg daily intake.

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α-Aminosulfonic Acids and Viral Propagation.* (19624)

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In the case of methionine(1-3) and tryptophane(4), it has been shown that metabolites as small as these amino acids are involved in the propagation of animal viruses and that analogs of these compounds will inhibit viral multiplication. A number of interesting analogs of amino acids have been prepared by McIlwain. *In vitro* these α-aminosulfonic acids are potent inhibitors of bacterial growth (5). Further α-aminophenylmethanesulfonic acid and α-aminoisobutanesulfonic acid which resemble in structure phenylalanine and valine have been reported to inhibit the multiplication of vaccinia virus in tissue culture(6). An analog of glycine, α-aminomethanesulfonic acid, will inhibit the propagation of a bacteriophage of *B. coli*(7). The mode of action of these compounds to the present has not been clearly explained, but there is evidence to show that they interfere with amino acid metabolism.

Three α-aminosulfonic acids reminiscent in structure of tyrosine or phenylalanine have been synthesized and tested as inhibitors of viral propagation in various biological systems. In the following, the results of these studies are reported, and their significance is discussed.

Virus and tissue. The PR8 strain of Type A influenza virus was selected for those studies in which the Warburg flask culture or the embryonate egg was employed. After isolation from man, this virus had undergone 7 passages in ferrets, 593 passages in mice, and 131 passages in eggs. For the experiments performed with mice, the WS strain of Type A influenza virus was used. This virus was cultured for several years in tissue culture and has undergone 99 passages in mice(8). The host cells for the Warburg culture were those of the chorioallantoic membrane and were

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TABLE I. Inhibition of the Propagation of Influenza Virus in Tissue Culture by Aminosulfonic Acids. Duration of each exp. approximately 26 hr; initial titer* in each exp. approximately 2.7 logs.

Inhibitor	α-amino-*p*-methoxyphenylsulfonic acids					
Molar conc. on × 10^4	0	1.4	2.8	5.7	8.5	14
Final egg infectivity titer*	8.5	7.7	6.5	5.5	3.5	2.7
Inhibitor	α-aminophenylmethanesulfonic acid					
Molar conc. × 10^4	0	.8	1.6	3.2	6.4	9.6
Final egg infectivity titer	8	7.3	6.5	6	5.5	2.3
Inhibitor	α-amino-β-phenylethanesulfonic acid					
Molar conc. × 10^4	0	.7	1.5	3	6	9
Final egg infectivity titer	9	7.7	6.3	5.8	3.7	1.5

* Titers expressed as the log of the reciprocal of the dilution.

TABLE II. Inhibition of Propagation of Influenza Virus in the Embryonate Egg by Aminosulfonic Acids. Duration of the experiment was 28 hr. Each egg received an inoculum of .1 ml of virus of titer‡ 2.

		Inhibitor conc., mg/egg									
		0	2	4	6	8	12	16	20	24	28
A*	Fraction of eggs dead	0/10	0/10	0/10	0/10	0/10	0/10	1/10	1/10	0/10	3/10
	" " " with RBCT†	10/10	4/10	4/10	1/10	0/10	0/10	0/10	0/10	0/10	0/10
	Egg infectivity titer‡	9.2	8	7.6	6.4	.5	0	0	0	0	0
B*	Fraction of eggs dead	0/10	0/10	0/10	1/10	0/10	1/10	3/10	3/10	3/10	4/10
	" " " with RBCT†	10/10	9/10	7/10	3/10	0/10	0/9	0/10	0/10	0/10	0/10
	Egg infectivity titer‡	9.7	9.5	9.5	8.5	1.5	.5	2.3	0	0	0
C*	Fraction of eggs dead	1/10	0/10	1/10	2/10	0/10	4/10	5/10	7/10	8/10	9/10
	" " " with RBCT†	9/10	5/10	5/10	1/10	0/10	0/10	0/10	0/10	0/10	0/10
	Egg infectivity titer‡	9.7	9.7	9.3	8.3	4.8	0	0	—	—	—

* A = α-amino-*p*-methoxyphenylmethanesulfonic acid. B = α-aminophenylmethanesulfonic acid. C = α-amino-β-phenylethanesulfonic acid.

† RBCT—positive hemagglutinin. ‡ Titer expressed as the log of the reciprocal of the dilution.

TABLE III. Effect of α-amino-*p*-methoxyphenylmethanesulfonic Acid on Respiration of the Chorio-Allantoic Membrane. Duration of exp. 28 hr.

Molar conc. of aminosulfonic acid × 10^4	Oxygen uptake*	Final virus titer egg infectivity† (initial = 3)
0	21.6	7.3
2.8	20.9	5.7
5.7	18.9	4.7
8.5	19.8	2.7

* μl of oxygen/200 mg of tissue/30 min.

† Titer expressed as the log of the reciprocal of the dilution.

obtained from 14-day-old embryonate chicken eggs. *Warburg flask culture.* In some experiments the host-virus system was maintained in the flasks of a Warburg apparatus using a modified Simms' medium. The details of this method have been reported previously(1,9). Each flask contained 200 mg of chorioallantoic membrane which was used intact without mincing. The cultures were incubated at 37°C with shaking, and the duration of each experiment is given in the corresponding tables. The inoculum was prepared by making suitable dilutions of infected allantoic fluid in Simms' solution. In all instances 0.3 ml of inoculum was used per flask, and the final volume of fluid and tissue was 3.0 ml. *Virus titer.* The amount of virus was estimated by determining the infectious titer for eggs. For this purpose 10-fold serial dilutions of the virus were prepared in broth and 4 eggs were inoculated with 0.1 ml of each dilution. After 3 days of incubation at 37°C samples of allantoic fluid were removed from each egg. These fluids were tested for virus by the addition of red blood cells. The 50% infectious titer was calculated using the method of Reed and Muench(10). Virus titers were also estimated by red cell agglutination using a pattern method(11). *Oxygen uptake.* The respiration of the cultures was measured manometrically in the conventional manner with the Warburg apparatus. *Compounds tested.* α-Aminophenylmethanesulfonic acid was prepared by the method of McIlwain(5). α-

Amino-p-methoxyphenylmethanesulfonic acid was prepared in this laboratory from anisaldehyde using the procedure described by McIlwain for the synthesis of α-aminoisobutanesulfonic acid. The product was recrystallized from ethanol and water. Analysis calculated for the monohydrate: $C_8H_{11}O_4SN \cdot H_2O$

	Calculated	Found
C	40.84	41.76
H	5.58	5.58
N	5.95	5.87

The author is indebted to Parke, Davis and Company for supplying a sample of α-aminophenylethanesulfonic acid and for supplying amounts of the other analogs in sufficient quantity for animal testing.

Results. Effect of aminosulfonic acids on influenza virus in vitro. Prior to testing the effect of the aminosulfonic acids on the host-virus system, it was desirable to know what interaction such organic structures might have with the virus alone. Since these compounds act similarly and have the same reactive groups, only data for α-amino-p-methoxyphenylmethanesulfonic acid will be presented. Various concentrations of this sulfonic acid were prepared in 0.15 M saline. After the pH had been adjusted to 7.0, the preparations were added to equal volumes of allantoic fluid containing influenza virus. Control samples were made by mixing equal amounts of infected allantoic fluid and isotonic saline. Some of the mixtures were incubated for 18 hours at 4°C and others for the same duration at 37°C. At the end of the incubation period, the titers of infectivity for eggs and of hemagglutination for red blood cells were determined. The maximum concentration of inhibitor used, 8.5×10^{-3} molar, was found to have no effect on the infectivity or hemagglutinating properties of the virus. This demonstrates that the sulfonic acid is not virucidal under these conditions in that it does not destroy influenza virus on contact.

Effect of aminosulfonic acids on the propagation of influenza virus in tissue culture. In 3 separate experiments the multiplication of influenza virus in Warburg flask cultures was estimated in the presence and absence of various concentrations of aminosulfonic acids. The inhibitors, the virus, and the tissue were added in that order, while the flasks were immersed in an ice bath. When all additions were completed, the flasks were transferred to the water bath at 37°C. Each of the compounds was found to be inhibitory at approximately the same concentration. α-amino-β-phenylethanesulfonic acid produced inhibition at slightly lower concentrations than did the other analogs. This sulfonic acid at a 9.0×10^{-4} molar concentration completely suppressed viral multiplication and showed some inhibitory effect at concentrations as low as 0.7×10^{-4} molar (Table I). The ability to recover from a completely inhibited culture the virus which was added in an inoculum (Table I), is further evidence that the action of these sulfonic acids is not virucidal.

Effect of aminosulfonic acids on influenza virus propagation in the embryonate egg. In view of the antiviral activity of these inhibitors in tissue culture (Table II), their effect on the propagation of virus in the embryonate egg was studied. Ten eggs, which were employed for each concentration of inhibitor tested, were inoculated with influenza virus via the allantoic route. Fifteen minutes later appropriate dilutions of the aminosulfonic acids, prepared at pH 7.0 in saline, were injected at the same site. After an incubation period of 28 hours, the eggs were chilled and samples of allantoic fluid were removed. The fraction of eggs which died during the incubation period and the fraction with hemagglutinin were recorded. The viral infectivity for eggs was determined on samples of allantoic fluid pooled from each group. These data for each aminosulfonic acid are recorded in Table II. The 50% lethal dose per egg of α-amino-p-methoxyphenylmethane sulfonic acid was approximately 28 mg. Concentrations of this analog as low as 4 mg per egg showed some inhibitory effect on the yield of virus produced. When 8 mg of the compound were employed, complete inhibition of the formation of hemagglutinin in each egg was observed. Under the same conditions, the infectivity titer was $10^{-0.5}$, while that of the control was $10^{-9.2}$. This compound is of particular interest, since the amount which shows toxicity for the embryo is about 6 times that which will inhibit the multiplication of influenza virus in the embryonate egg (Table II). The other aminosulfonic acids produced similar results. However, α-amino-β-phenylethanesulfonic acid was definitely more toxic to the embryo than either substituted methanesulfonic acid. The increased toxicity for the host was not paralleled by increased anti-viral activity (Table II).

Effect of aminosulfonic acids on influenza virus propagation in the brains of mice. Eighty mice were inoculated intracerebrally with the WS strain of Type A influenza virus. Three groups of 20 mice were inoculated by the same route one hour later with 300 μg of one of the 3 aminosulfonic acids, while one group was inoculated with saline. Eighty-five per cent of the control animals died, and similar results were obtained with 2 of the analogs. Of the group of mice receiving α-amino-β-phenylethanesulfonic acid only 45% succumbed. In a series of 3 similar experiments 15, 13, and 20% of the control animals survived, while 55, 52, and 56% of those treated with the ethanesulfonic acid derivative survived. The compound was without effect when administered intraperitoneally or when incorporated into the diet. It did not affect the survival of animals receiving an inoculum of virus which produced an incidence of death much higher than 85%.

Concerning the mode of action of the aminosulfonic acids. The α-aminosulfonic acids are not stable structures in aqueous solvent at elevated temperatures(5). Hence, it was considered that their antiviral activity might be due to the aldehydes which are formed by decomposition. Anisaldehyde, which results from the decomposition of α-amino-*p*-methoxyphenylmethanesulfonic acid, was tested in tissue culture and found to be inactive at 12 x 10^{-4} molar concentration. This amount of the corresponding aminosulfonic acid will produce complete inhibition (Tables I and II). Further, the aldehyde which showed no antiviral activity was found to be quite toxic for the embryonate egg. Ammonium sulfate and sodium bisulfite were both tested in eggs and found to be inactive at concentrations corresponding to effective inhibitory levels of the aminosulfonic acids. Thus, the decomposition products cannot account for the antiviral activity of this sulfonic acid. However, their instability may explain the difference in toxicity of the 3 aminosulfonic acids for the host, since the most toxic of the compounds (α-amino-β-phenylethanesulfonic acid) also appears to be the least stable of the 3 inhibitors.

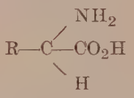
In view of the structural relation of the sulfonic acids to phenylalanine and tyrosine, attempts were made to prevent or reverse their inhibitory action in tissue culture by the use of the corresponding aromatic amino acids. All attempts in this direction were without success. The action of 5.7 x 10^{-4} molar α-amino-*p*-methoxyphenylmethanesulfonic acid could not be prevented by the addition of 10 times that amount of tyrosine or phenylalanine.

A number of compounds which interfere with the oxidative processes of the Krebs cycle inhibit viral multiplication(9,12). These aminosulfonic acids apparently do not act via that principle, since the respiration of the chorioallantoic membrane is unaltered by concentrations of α-amino-*p*-methoxyphenylmethanesulfonic acid which produced complete inhibition of viral propagation (Table III). Since the endogenous respiration of tissue will not continue in the medium if the cellular membranes are disrupted, no gross damage to the host cells seems to be involved when they are maintained in the presence of the compound. This conclusion is further supported by the low toxicity *in vivo* of the sulfonic acid for the embryonate egg (Table II).

Discussion. The action of these α-aminosulfonic acids is not virucidal. Rather, the effect is directed toward some site of the host cell which is essential for the viral synthetic process. That this is a desirable site for chemotherapeutic action is indicated by the low toxicity of amounts of these compounds which cause inhibition of viral propagation in the egg (Table II).

The inability of ammonium ions, bisulfite ions, and the aldehyde precursor to inhibit demonstrates the essentiality of the intact α-aminosulfonic acid structure for anti-viral activity. In contrast the second substituent on the α-carbon can be varied considerably without a resulting quantitative alteration of the inhibition produced in "closed" biological systems (the embryonate egg and tissue culture).

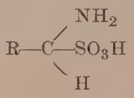
The results obtained with these analogs are intelligible, if one assumes that their effect is directed toward an enzyme which has only a limited specificity for its substrate. Certain enzymes possess only limited specificity in that they act upon a number of different metabolites. For example the d- and l-amino acid oxidases are specific for α-amino acids, but their activity is not confined to any particular amino acid. Such enzymes may be quite specific for certain functional groups or parts of the substrate, presumably those groups which are common to all susceptible substrates. In the example of the α-amino acid oxidase, it is the α-amino carboxylic acid group which is required.



$$R{-}C(NH_2)(H){-}CO_2H$$

For this type of enzyme where the enzyme-specific grouping of the substrate is known, it should be possible to design a variety of inhibitors. They would be composed of 2 elements. One would have the geometric and electronic specifications necessary for enzymatic association and would be an analog of the enzyme-specific portion of the metabolite. The second element would be a non-enzyme-specific portion corresponding to the R moiety of the amino acid. Thus, modifications in the R grouping would lead to a number of inhibitors each capable of action at the enzyme site, but greatly differing in physical properties.

McIlwain reported the synthesis of a series of α-aminosulfonic acids which were found to be potent inhibitors of bacterial growth(5).



$$R{-}C(NH_2)(H){-}SO_3H$$

Further, the effect of these various analogs was specific only in that it was confined to action on the amino acids. Those which were most effective in alleviating the inhibition produced by any particular α-aminosulfonic acid did not necessarily correspond to the amino acids which bore the closest structural relation to the analog. These findings may be consistent with the concept outlined above. The α-aminosulfonic acids may act upon a single enzyme in the cell which normally has multiple substrates. It is not likely that such an inhibition would be completely reversed by any single substrate.

From the viewpoint of chemotherapy a useful inhibitor must possess, in addition to the proper geometry, a number of chemical and physical properties associated with permeability and solubility. Frequently, it is difficult to alter these physical properties by making structural changes without affecting the essential geometrical specifications. Hence, it may be more feasible to design useful inhibitors for those enzymes which possess only limited substrate specificity than for those with a higher degree of substrate selectivity.

Summary. Three α-aminosulfonic acids (α-aminophenylmethanesulfonic acid, α-amino-β-phenylethanesulfonic acid, and α-amino-*p*-methoxyphenylmethanesulfonic acid) were prepared and tested as inhibitors of the propagation of influenza virus. Each was found to cause marked inhibition of viral synthesis in tissue culture at a concentration of 5 x 10^{-4} molar. In the embryonate egg 8 mg of each compound completely inhibited the multiplication of influenza virus, although the 50% lethal dose for eggs of α-amino-*p*-methoxyphenylmethanesulfonic acid was approximately 30 mg per egg. Under special conditions, α-amino-β-phenylethanesulfonic acid afforded some protection to mice which were infected with the WS strain of Type A influenza virus. These sulfonic acids are not virucidal, and they do not affect the respiration of tissues cultured in the Warburg flask and are not reversed by tyrosine nor phenylalanine to which they are structurally related. Ammonium ions, bisulfite ions, and *p*-methoxybenzaldehyde did not affect viral propagation. A possible mode of action of these amino acid analogs is discussed.

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Antimalarial Activities of Triazine Metabolites of Chlorguanide and Dichlorguanide.* (19625)

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During a study of the metabolic fate of the antimalarial drug chlorguanide, Carrington and coworkers(1) isolated a compound from the urine of humans and the feces of rabbits which was believed to be the hypothetical "active metabolite"(2-4) of the above drug. The isolated compound, tentatively identified as 2,4-diamino-1-p-chlorophenyl-1,6-dihydro-6,6-dimethyl-1,3,5-triazine (*CPT*), was reportedly 10 times as active as chlorguanide against infections with *Plasmodium gallinaceum* in the chick. These workers also described a second compound, 2,4-diamino-1-(3, 4-dichlorophenyl)-1, 6-dihydro-6, 6-dimethyl-1,3,5-triazine (*DCPT*), which was considered (5) to be the corresponding metabolite of dichlorguanide(6) and which was 50 times as active as the latter drug and 100 times as active as chlorguanide against infections with the above plasmodium(1,5).†

These findings were of considerable interest to this laboratory, which since 1946 has studied the metabolic fate of chlorguanide and related biguanides in man and rhesus monkey. Although this investigation(8,9) had pointed to the pattern of metabolic degradation described by Carrington, it had failed to provide any evidence for the presence in urine, feces, sera or tissues of metabolites more active than their parent drugs. Since this conclusion was based on assessments of activity in *P. cynomolgi* infections in the rhesus monkey(9), whereas that of Carrington and Davey was based on work with *P. gallinaceum*, the question arose whether the divergent findings were related to the use of different test objects. This led to a study of the activities of the triazines, *CPT* and *DCPT*, against infections with *P. cynomolgi*, the results of which are presented in this report.

Materials and methods. The comparative antimalarial activities of *CPT* and chlorguanide were measured in a group of 18 rhesus monkeys (3.5 to 4.8 kg), each of which was inoculated intravenously with approximately 500,000 trophozoites, obtained by suitable dilution in 0.85% saline of the blood of a passage monkey infected with the stock strain of *P. cynomolgi*.‡ Through use of conventional thick and thin blood films stained with Giemsa, parasitemias were followed daily until a density of approximately 100 parasites per 10,000 erythrocytes was attained. At this point, groups of 8 monkeys were treated either with *CPT* or with chlorguanide, the 2 remaining animals serving as untreated controls. Because previous studies(9) had shown that absorption of orally administered dihydrotriazines was uncertain, each drug was adminis-

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† The activities of these triazines against *P. gallinaceum* infections have been confirmed recently by Coatney(7).

‡ The strain of *P. cynomolgi* was obtained June 1946 from Dr. R. J. Porter, University of Michigan. Since that time it has been maintained by serial intravenous trophozoite passages through untreated rhesus monkeys at intervals of 2 to 4 weeks.

tered to 4 monkeys by stomach tube and to 4 intramuscularly. Solutions of these drugs in 0.85% saline were employed for all treatments. These solutions were prepared immediately prior to administration in order to eliminate the problem of triazine rearrangement (1,5,9). The doses of chlorguanide used were arranged on the basis of previous large-scale experience(10,11) to provide subeffective, minimally-effective, parasitemia-suppressive, and curative dosage levels. Identical doses of *CPT* were employed in order to detect either enhanced or reduced activity. In all instances the drugs were administered once daily (8:30 A.M.) for 7 consecutive days. Parasitological studies were made daily throughout treatment and for at least 10 days thereafter, unless parasitemias were abolished. In the latter instance, blood examinations were made once weekly either until a recrudescence occurred or until evidence was obtained that the infection had been cured.[§] The study of the comparative activities of *DCPT* and chlorguanide followed the above pattern in all important respects. The only differences were the use of 20 monkeys instead of 18, to provide 4 untreated controls, and the administration of smaller doses of *DCPT* than chlorguanide, in order to detect the enhanced activity of the dichlorguanide metabolite anticipated from the results of the avian studies of Carrington and Davey(1,5).[‖]

Results. The data on the relative activities of *CPT* and chlorguanide, summarized in Table I, indicate clearly that the parent biguanide is more effective than its metabolite against infections with *P. cynomolgi.* The small number of monkeys used in this study makes it impossible to appraise this activity difference precisely. However, a rough comparison, based on the doses of the respective drugs required to effect minimal but significant reductions in parasitemia, suggests that chlorguanide is at least twice and possibly 4 times as active as *CPT*. Thus when administered intramuscularly, 0.075 mg per kg doses of *CPT* were required for the same effects as were produced by 0.0188 mg per kg doses of chlorguanide (cf. M-4872 and M-4892). Administered orally, 0.3 mg doses of *CPT* and 0.075 mg doses of chlorguanide gave comparable results (cf. M-4874 and M-4888).

The data in Table I also show that *DCPT* was somewhat more active than *CPT*. However, the activity of *DCPT,* the dichlorguanide metabolite, was no greater than that of chlorguanide. This is shown clearly by a comparison of the doses of these compounds required for minimal but significant effects on parasitemia. These doses were identical for the two compounds, 0.0188 mg per kg, intramuscularly (cf. M-4745 and M-4730) and 0.075 mg per kg, orally (cf. M-4775 and M-4726).

Discussion. From the data presented above

[§] Infections were considered cured when there was no recurrence of parasitemia within 12 weeks after treatment despite attempted provocation of relapse by splenectomy.

[‖] Since the triazine metabolites, *CPT* and *DCPT,* are not generally available and since their structures have not been established conclusively, it is important to record the sources and characteristics of the compounds used in this study. *DCPT* was obtained through the generosity of Dr. D. G. Davey, Biological Laboratories, Imperial Chemical (Pharmaceuticals) Ltd., Wilmslow, England. This compound was supplied as the monohydrochloride, m.p. 190° uncorrected. *Analysis.* Calculated for $C_{11}H_{13}Cl_2N_5 \cdot HCl \cdot$ C, 40.95; H. 4.37; Cl, 32.97; N, 21.71. Found: C, 41.39; H, 4.21; Cl, 32.77; N, 21.22. According to ultraviolet absorption measurements(1,9) this substance was converted quantitatively to 4-amino-2-(3,4-dichloroanilino)-1,6-dihydro-6, 6-dimethyl-1,3,5-triazine by heating in 2 N sodium hydroxide at 100° for 20 minutes. The hydrated dihydrochloride of *CPT* was synthesized in our laboratories(12) by refluxing a concentrated hydrochloric acid solution of p-chlorophenylbiguanide with acetone, m.p. 216-21°, uncorrected. *Analysis.* Calculated for $C_{11}H_{14}ClN_5 \cdot 2\ HCl \cdot 2\ H_2O$: N, 19.42. Found: N, 19.94. The monohydrochloride (m.p. 200-207°) and picrate (m.p. 215-218°) of *CPT* were also prepared. *Analysis.* Calculated for $C_{11}H_{14}ClN_5 \cdot HCl$: C, 45.84; H, 5.25. Found: C, 45.64; H, 5.09. Calculated for $C_{11}H_{14}ClN_5 \cdot C_6H_3N_3O_7$: C, 42.46; H, 3.56; N, 23.31. Found: C, 42.72; H, 3.13; N, 22.68. Ultraviolet absorption studies showed that *CPT,* like *DCPT,* was converted quantitatively to the corresponding anilino isomer (4-amino-2-p-chloroanilino-1, 6-dihydro-6,6-dimethyl-1,3,5-triazine) by heating in 2 N sodium hydroxide at 100° for 20 minutes(1,9). The chlorguanide monohydrochloride used in current experiments was provided by Dr. Davey in 1946. According to countercurrent distribution pattern, the homogeneity of this preparation was in excess of 98%.

TABLE I. Comparative Antimalarial Activities of Chlorguanide, 2,4-diamino-1-p-chlorophenyl-1,6-dihydro-6,6-dimethyl-1,3,5-triazine (*CPT*), and 2,4-diamino-1-(3,4-dichlorophenyl)-1, 6-dihydro-6, 6-dimethyl-1,3,5-triazine (*DCPT*).

Drug and route of adminis.	Monkey No.	Daily dose, mg base/kg body wt	No. parasites/10000 erythrocytes											Subsequent course of infection†
			Day of treatment							Day after treatment				
			0	1	2	3	4	5	6	1	2	3	4	
CPT, intramusc.	4865	.0047	120	84	370	540	1010	600	130	62	34	16	4	P
	78	.0188	200	360	1700	2100	1240	200	96	55	67	120	160	P
	72	.075	75	59	51	47*	56*	75*	21*	1	<1	<1	<1	R
	67	.3	140	150*	82*	1*	2	2	1	1	—	—	—	R
Chlorguanide, intramusc.	87	.0047	220	390	1390	1430	930	310	170	48	88	63	73	P
	92	.0188	150	230	420	400*	300*	180*	49*	8*	3	2	1	P
	96	.075	190	160*	120*	4*	6*	1	2	<1	—	—	<1	R
	98	.3	180	140*	54*	4*	7*	5*	3*	<1	—	—	—	C
CPT, oral	80	.0188	110	220	1120	1010	770	210	460	280	200	140	110	P
	71	.075	170	360	660	860	730	320	110	37	27	23	91	P
	74	.3	86	92*	18*	3*	1	1	1	<1	—	<1	<1	R
	84	1.25	120	110	47*	4*	10*	1	1	<1	—	—	—	C
Chlorguanide, oral	69	.0188	130	190	350	420	250	160	170	30	30	10	10	P
	88	.075	130	170	210*	120*	32*	14*	4*	1	2	<1	<1	R
	89	.3	110	100*	69*	5*	1*	2	1	<1	<1	—	—	R
	77	1.25	170	180*	120*	9*	6*	3*	2	2	—	—	—	C
Controls	63	0	110	170	200	610	550	440	190	43	18	15	7	
	76	0	79	190	600	1330	780	420	360	59	46	45	86	
DCPT, intramusc.	4770	.0012	82	260	420	900	1130	830	530	320	130	120	70	P
	32	.0047	110	230	760	1010	650	240	67	40	77	96	140	P
	45	.0188	81	120*	45*	5*	7*	1	1	5	15	19	99	P
	73	.075	130	80*	17*	6*	1*	1*	1	<1	—	—	—	R
Chlorguanide, intramusc.	19	.0047	56	140	740	440	520	98	66	16	59	59	31	P
	30	.0188	14	110	420*	120*	150*	7*	5*	<1	<1	<1	<1	R
	17	.075	8	18	22*	6*	4*	3*	<1	<1	<1	<1	<1	R
	34	.3	11	59	87*	7*	4*	2*	2*	<1	<1	<1	—	C
DCPT, oral	60	.0047	120	520	1130	2100	1650	750	270	59	45	130	100	P
	80	.0188	160	420	1340	1390	1570	830	140	85	69	150	320	P
	75	.075	130	330	460	260*	100*	36*	3*	3*	1*	<1	<1	R
	71	.3	110	98*	52*	5*	2*	3*	2*	<1	<1	2	7	R
Chlorguanide, oral	41	.0188	17	100	580	1050	950	490	140	62	65	150	190	P
	26	.075	13	25	19*	9*	4*	2*	2*	<1	<1	<1	<1	R
	20	.30	82	120	57*	12*	10*	8*	3*	<1	<1	<1	<1	R
	38	1.25	66	120	170*	8*	3*	1*	<1	<1	—	—	—	C
Controls	43	0	55	82	940	1200	1080	380	170	7	32	20	47	
	52	0	160	600	820	1610	1470	630	85	86	92	120	160	
	68	0	86	320	770	1340	1140	550	170	84	100	91	170	
	82	0	20	25	300	330	650	630	950	370	230	15	10	

* Abnormal schizonts, with chromatin scattered diffusely throughout parasite, predominate.

† P = infection persisted, following pattern of untreated infection; R = spontaneous recrudescence of infection; C = cure, or eradication, evidenced by absence of parasitemia for 12 wk despite splenectomy.

it is evident that the activities of the triazine metabolites of chlorguanide and dichlorguanide against *P. cynomolgi* infections in the rhesus monkey are in no way comparable to the activities of these compounds against *P. gallinaceum* infections in the chick. Whereas in this avian disease *CPT* and *DCPT* were respectively 10 and 100 times as effective as chlorguanide, in the simian infection *CPT* had only one-half to one-fourth the activity of the parent biguanide, *DCPT* equal activity. At present there is no satisfactory explanation for the divergent responses of the above infections. Such diverse reactions are not uncommon, however, in malaria chemotherapy(13). In this instance, they may rest either on inherent differences in the susceptibilities of *P. cynomolgi* and *P. gallinaceum* to the specific compounds, or to differences in the physiological disposition of the metabolites in the chick and monkey, the former possibility being the more likely. In any case, the demonstration that *CPT* is distinctly less active than chlorguanide against infections with *P. cynomolgi* explains previous failures to isolate the hypothetical "active metabolite" of chlorguanide.

In conclusion some comment on the practical implications of the current findings should be made. The high activities of CPT and especially *DCPT* against infections with *P. gallinaceum* might suggest that these drugs would be a striking improvement over chlorguanide in the treatment of human malaria. Such optimism is not justified by the findings in *P. cynomolgi* infections, which in a wide experience have proved to be a highly reliable indicator of the value of drugs against human malaria. The demonstrations that in the simian disease *CPT* is distinctly less effective than chlorguanide and that *DCPT* has essentially the same effectiveness as this biguanide suggest that with respect to inherent antimalarial activity these metabolites would have little to offer in the treatment of human malaria.

Summary. The activities of the triazine metabolites of chlorguanide and dichlorguanide have been studied in *P. cynomolgi* infections in the rhesus monkey. The results show that the chlorguanide metabolite, *CPT*, has only one-half to one-fourth the activity of the parent drug, whereas the dichlorguanide metabolite, *DCPT*, has activity equal to that of chlorguanide. These findings are in marked contrast to reported results on *P. gallinaceum* infections in the chick, against which *CPT* and *DCPT* are respectively 10 and 100 times as effective as chlorguanide.

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Occurrence of Acute Pulmonary Edema in Experimental Alkalosis. (19626)

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In previous studies of experimentally induced tetany(1,2), it was found that venoclysis of solutions of sodium carbonate and sodium bicarbonate for the production of alkalotic tetany in cats frequently resulted in the appearance of gross lung edema. This edema resembled that produced by the administration of toxic doses of ammonium salts previously described and studied by several of us(3,4). The purpose of this report is to describe the experimental production of acute pulmonary edema with the alkaline salts of sodium together with some preliminary observations on the mechanism of its production.

Material and methods. The data presented in this paper are based on the study of 25 adult cats anesthetized with sodium pentobarbital (35 mg/kg). The trachea was cannulated in all experiments. In a number of experiments artificial respiration was provided through the tracheal cannula as positive pressure blasts from a Harvard type respirator pump. Alkalosis was produced in 20 cats by the continuous infusion of a 5% solution of sodium carbonate or an 8% solution of sodium bicarbonate into the femoral vein. From time to time venous blood was collected under mineral oil in a hypodermic syringe and the pH determined with the Beckman glass electrode pH meter. Hypocalcemic tetany was produced in 4 cats by venoclysis of a phosphate buffer with a pH of approximately 7.4 (3 g of monobasic sodium phosphate, 30 g of dibasic sodium phosphate in 600 cc of solution). In the fifth cat, hypocalcemic tetany was produced by venoclysis of a 2% sodium citrate. The rate of infusion was adjusted so that manifest tetany was present from 10 to 20 minutes and then slowed to maintain a steady state of tetany until the termination of the experiment. In 5 experiments the cats were pretreated with dibenamine (25 mg/kg in 2 cats, 40 mg/kg in 3 cats by intraperitoneal administration), an adrenergic blocking agent, 3 to 4 hours before the administration of sodium carbonate. Analysis of edema fluid for total protein content was carried out in 5 experiments with a modification of the Koch-McMeekin micro-Kjeldahl method(5) for nitrogen. Postmortem examination was performed with special reference to the thoracic viscera in all experiments. In a number of experiments a lobe of lung was ligated and fixed by immersion in 10% formalin, sectioned and stained with hematoxylin and eosin for microscopic examination.

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Results. The characteristics of the tetany produced by this method have been described elsewhere(2). Seven cats were given an 8% solution of sodium bicarbonate by venoclysis. Of this group, 5 cats received 50 to 100 cc of solution over a period ranging from 20 to 100 minutes. The blood pH was raised from a normal of 7.45 ± 0.05 to 7.85-7.95. Gross pulmonary edema appeared in all 5 experiments. The remaining 2 cats were given 40 and 45 cc respectively of the sodium bicarbonate solution over a period of 2 hours. The pH of their blood did not exceed 7.75 and the degree of tetany developed was moderate. Pulmonary edema did not occur in these 2 animals.

Eight cats were given 35 to 100 cc of a 5% solution of sodium carbonate by venoclysis over periods ranging from 25 minutes to 120 minutes. Six of the 8 cats developed acute pulmonary edema. The pH of the blood was raised to 7.80 – 7.90 or more in these animals. Artificial respiration did not appear to influence the production of lung edema. The hypocalcemic tetany produced in four cats by venoclysis of a phosphate buffer, in one by a 2% solution of sodium citrate was not accompanied by pulmonary edema.

Because of the efficacy of dibenamine in preventing the lung edema produced by ammonium salts(4), dibenamine was employed in this study. Of five cats treated with dibenamine, four developed lung edema in the usual fashion upon administration of 5% sodium carbonate. The presence of the full effect of dibenamine was demonstrated in one of these animals by injection of 0.5 cc of a 1:10,000 solution of epinephrine intravenously, which produced a marked reduction in arterial pressure instead of the usual elevation.

In most of these experiments, the presence of lung edema first made itself known by the appearance of edema fluid in the tracheal cannula. This fluid was usually straw-colored and clear with a variable amount of foam, although occasionally the fluid was blood-tinged. Analyses for total protein carried out on samples of edema fluid collected in 3 experiments yielded the following: 0.8%, 1.0% and 1.4%. Edema fluid in 2 experiments in which animals were pretreated with dibenamine contained 4.6% and 4.9% protein. Upon autopsy and microscopic examination the lungs had the usual appearance of acute pulmonary edema with areas of congestion and hemorrhage.

Discussion. The administration of solutions of sodium bicarbonate and sodium carbonate by venoclysis in cats results quite consistently in the appearance of acute pulmonary edema. This phenomenon seems to have escaped notice. Other routes of administration for the sodium salts and other animal species have not been studied. The lung edema does not appear to be related to the tetany which is present in these animals, but rather to the elevation in blood pH. Hypocalcemic tetany produced by venoclysis of solutions of phosphate or sodium citrate is indistinguishable from alkalotic tetany but does not eventuate in pulmonary edema. In our experience, the blood pH has to exceed 7.80 and has to be maintained for five minutes or longer before lung edema appears. Artificial respiration with positive pressure does not seem to inhibit this form of lung edema, although it has been reported to inhibit other types of experimental lung edema(6).

The relatively low protein content of the edema fluid indicates that a significant increase in alveolar capillary permeability was not present at the time the fluid was formed. However, the anoxia which follows the obstruction of alveolar spaces and the duct system by edema fluid quickly produces an increase in capillary permeability allowing an appreciable leakage of plasma proteins and often culminating in the rupture of capillary walls with consequent hemorrhage. The high protein content of the edema fluid in animals pretreated with dibenamine may be attributable to a more fulminating onset of lung edema with earlier anoxia. These results indicate that an increase in alveolar capillary permeability is not involved in the initiation of lung edema in alkalosis. The acute lung edema occurring in alkalosis resembles that produced by ammonium salts. Both are preceded by and associated with abnormal neuromuscular phenomena, tetany in the former and tonic and clonic convulsions in the latter (3) and the protein content of the edema fluid is relatively low(4). However, dibenamine, which blocks the excitor effects of sympathetic nerve impulses at the effector organs(7), is exceedingly effective in preventing ammonium lung edema(4), but is quite ineffectual in alkalosis lung edema. An understanding of the pathogenesis of this form of acute pulmonary edema awaits further investigation.

Summary. 1. Venoclysis of solutions of sodium carbonate and sodium bicarbonate in quantities sufficient to produce a severe alkalosis and tetany produced acute pulmonary edema in most cats. 2. This lung edema was not inhibited by artificial respiration nor by pretreatment with dibenamine. 3. The edema fluid contained very small concentrations of proteins, ranging from 0.8% to 1.4%. In two animals that had been pretreated with dibenamine the total protein approached 5%. 4. Severe hypocalcemic tetany produced by venoclysis of a phosphate buffer and citrate solution did not result in lung edema.

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Nature of an Accelerator of Prothrombin Activation Arising During Storage of Purified Prothrombin.* (19627)

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A most fruitful approach to the study of the mechanisms of blood coagulation has been the purification of some of its important components. For example, our laboratory has devoted much effort to the purification of prothrombin and to a study of its conversion to thrombin(1-4). Prothrombin has been separated from bovine plasma in sufficient quantity

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* Aided by a grant from the U. S. Public Health Service, National Institutes of Health.

and purity for scientific study. Recent work has shown that the same methods are also applicable to preparation of human prothrombin (5). Autocatalytic activation of prothrombin can be demonstrated by the use of such prothrombin preparations(2). For example, if prothrombin is dissolved in 25% sodium citrate solution and allowed to stand at room temperature, measurable amounts of thrombin are formed after about 5 hours. Thrombin forms thereafter at a rate which corresponds to that of an autocatalytic reaction. The rate of thrombin formation in such an experiment can be accelerated by the addition of small amounts of purified thrombin. In a series of fundamental studies, various derivatives were made. One of these is obtained when thrombin is added to an aqueous solution of prothrombin. The derivative is insensitive to activation with calcium, Ac-globulin, and thromboplastin(6). This derivative may be identified, in part, by electrophoresis, because it has a lower mobility than prothrombin. Another inactive derivative of prothrombin may be formed by adding 3,4,4′-triaminodiphenyl sulfone to purified prothrombin in 25% sodium citrate solution(7). Under similar circumstances, but with 3-chloro-4,4′-diaminodiphenyl sulfone, an intermediate is produced during the conversion of prothrombin to thrombin(7). A derivative of prothrombin can also be formed by drying an aqueous solution of prothrombin from the frozen state and storing it in a desiccator(8). This preparation cannot be activated with biological activators but can be changed to thrombin in 25% sodium citrate solution by autocatalysis.

The present report concerns a previously unrecognized property associated with storage of aqueous solutions of purified prothrombin at approximately —10°C. Such solutions gradually lose prothrombin activity. It will be shown that this loss of prothrombin activity is accompanied by the development of another property; namely, the ability to accelerate the conversion of prothrombin to thrombin.

Methods. Preparation of prothrombin. Purified prothrombin was prepared by the methods of Ware and Seegers(1). It was freed from the last traces of Ac-globulin by heating the prothrombin to 53°C for 2 hours in solution in neutral distilled water. It was then dried by using cold acetone. When needed in the experiments some of the dehydrated prothrombin was dissolved in neutral distilled water so the resulting solution contained approximately 2000 units of prothrombin per ml. *Prothrombin analysis.* Prothrombin activity was measured by the modification of the original 2-stage method of Warner, Brinkhous, and Smith(9) suggested by Ware and Seegers(10). The modification consists of adding Ac-globulin to insure maximum prothrombin activation. *Thrombin analysis.* The method of Seegers and Smith(11) was used for the quantitative determination of thrombin. *Ac-globulin analysis.* Plasma Ac-globulin activity was determined by the method described by Ware and Seegers(12).

TABLE I. Changes Observed in Stored Frozen Purified Prothrombin Solutions.

Storage at −10°, days	Prothrombin, μ/ml	Thrombin, μ/ml	Ac-globulin, in bovine plasma, μ/ml
0	2000	0	120
4	2000	0	120
8	2000	0	120
12	2000	0	120
16	630	Trace	480
32	630	5	720

Experimental. A solution of purified prothrombin which was free from Ac-globulin was prepared by the method above. This solution was shown to be free from thrombin, and when it was used as a prothrombin source for the determination of the Ac-globulin content of the laboratory control bovine plasma, the result was the same as obtained for several years with the standard reagents in use for Ac-globulin analyses. This result was also the same as had been obtained for this plasma for several months with numerous lots of purified prothrombin. The solution of prothrombin was then stored at a temperature of approximately —10°C, and was kept in the frozen state for 32 days. During this time, samples were tested for prothrombin, thrombin, and Ac-globulin every 4 days, for 16 days, and again at 52 days. The analytical results are shown in Table I. This experiment was repeated often during the past 2 years.

The role of the increase in thrombin content in causing the apparent increase in Ac-globulin activity may be questioned. For use in Ac-globulin analyses, the prothrombin solution used is diluted to a final concentration of 1.34 units per ml. In this instance, the prothrombin concentration was 630 units per ml, and the thrombin concentration was 5 units per ml. The dilution employed was, therefore, 630/1.34 or 470 fold, resulting in a final concentration of thrombin only slightly higher than 0.01 unit per ml. During the course of nearly 5 years of experience with Ac-globulin analyses, it has been repeatedly observed that final thrombin concentrations as high as 0.02 unit per ml introduced with the prothrombin have no observable effect on Ac-globulin concentration determinations.

Further exploration of the meaning of the apparent increase in Ac-globulin activity indicated that the stored prothrombin contained an accelerator of prothrombin conversion. Many prothrombin preparations were studied and all of them were found to acquire this unusual property and to a certain extent, this seemed to be independent of the purity of the prothrombin. Further data on a typical preparation from this laboratory are given in Table II. The table shows the values obtained, a) with the standard prothrombin, b) a mixture of 3 parts of standard prothrombin and one part of stored frozen prothrombin, c) equal parts of the 2 prothrombin solutions, and d) the stored frozen prothrombin. In all of these analyses, the actual prothrombin concentration was the same by 2-stage analysis. The bovine plasma used was also the same in all instances so it must be concluded that the quantity of Ac-globulin was the same throughout and that the apparent differences in its concentrations are due to the quality of the prothrombin substrate used.

TABLE II. Effect of Prothrombin Substrate on Quantitative Bovine Plasma Ac-Globulin Analyses.

Prothrombin substrate*	Units Ac-globulin/ml
Stand. purified prothrombin	120
3 parts stand. prothrombin with 1 part stored frozen prothrombin†	250
2 parts stand. prothrombin with 2 parts stored frozen prothrombin†	399
Stored frozen prothrombin†	639

* Total prothrombin concentration was always standard for the procedure; namely, 1.34 units/ml.

† No. 510207.

Discussion. It is considered probable that the property described in the present report is possessed by a derivative of prothrombin. The acceleration of prothrombin conversion described does not occur with the freshly prepared prothrombin solution, nor does it occur as long as the prothrombin concentration, as measured by the use of activators of biological origin, remains unchanged. With the loss of prothrombin activity, however, the accelerator becomes detectable. These circumstances are compatible with the concept that the activity described is due to an alteration of the prothrombin molecule.

One may speculate that the prothrombin molecule is altered in such a way that it does not cause fibrinogen to coagulate, but that it will act in the same way as thrombin in autocatalysis. This would account for the loss of prothrombin activity associated with the gain of prothrombin conversion accelerator activity.

The present evidence, while compatible with the idea of development of a prothrombin derivative, does not exclude other interpretations. It is possible that the activity observed is due to a contaminant in the prothrombin preparations. This view requires that the contaminant becomes activated while the prothrombin loses its activity. That would be an unusual combination. If our estimates of the purity of the prothrombin preparations are correct, then an impurity could only be present in small quantities and its potency would have to be extraordinary.

Summary. Aqueous solutions of purified prothrombin, stored in a freezer at —10°C, lose appreciable activity over a period of a few weeks. Concomitantly, these solutions gain the ability to accelerate the conversion of prothrombin to thrombin, as evidenced by an apparent increase in Ac-globulin activity of plasma analyzed by using the stored solutions as the prothrombin substrate. It is believed that prothrombin itself becomes modified and acquires accelerator properties. The possibility of an impurity acquiring these properties

is not excluded, although that is considered unlikely.

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Effects of Beta Rays upon a Single Nerve Fiber. (19628)

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The histological changes of nerve fibers under severe irradiation by x-rays were reported by Janzen and Warren(1), and Davidoff *et al.*(2). Schmitz and Schaefer(3) were unable to demonstrate any changes of action potential of nerve fibers by irradiation. Audiat (4), Paulian(5), Rascanu(6), and others irradiated the nerve trunks and showed some changes of the action potential. It is the purpose of this report to deal with the effect of irradiation on the conduction of nerve impulse physiologically using a single nerve fiber of the sciatic gastrocnemius of the Japanese toad (*Bufo vulgaris*).

Methods. The source of beta rays was radon sealed in a glass tube (30 mg/cm^2 in thickness). The apparatus is shown schematically in Fig. 1. Two glass plates, covered with paraffin, carried parallel mica plates 10 μ thick that were insulated from each other. One of the mica plates had a small shelf, where the radon tube can rest at a distance of 1 mm from the nerve fiber. A single motor nerve-muscle sample of the sciatic gastrocnemius of toad, made by the method of Shimizu and Tasaki(7,8) and immersed in Ringer's solution, rested on the glass plates. Precautions were taken in handling to make a bridge of a single nerve fiber between the pools without exposing Ranvier's rings to air. The action current between insulated poles was measured by an oscillograph and recorded photographically.

Results. 1. *Changes of threshold value of stimulus by irradiation.* The value of threshold stimulation was measured before and after irradiation for short intervals. To properly express the dose received by the nerve fiber, roentgen equivalent physical (rep) should be the unit employed. However, since the con-

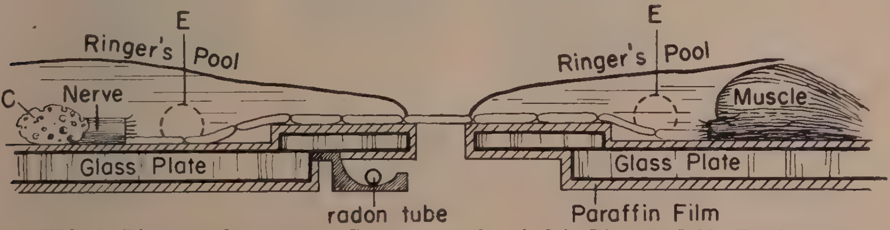

FIG. 1. Diagram of apparatus. C—cotton wool soaked in Ringer's fluid; E—electrode.

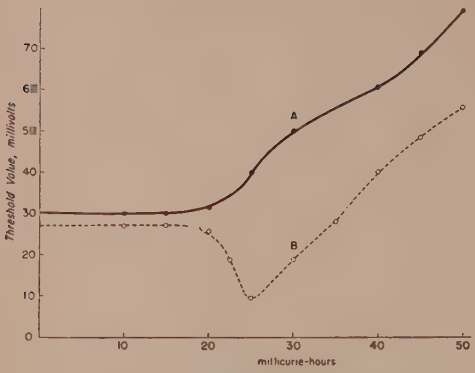

FIG. 2. Changes of threshold value of a single nerve fiber after irradiation. A—irradiated part; B—non-irradiated part.

ditions of the experiment such as filter and distance remained constant, the abscissa of Fig. 2 denotes relative values. The doses would be proportional to the millicurie-hours. The irradiation exposures are given in millicurie-hours, according to duration of exposure and intensity of the source. The decay of radon was taken into consideration. The ordinate indicates the value of threshold stimulus in millivolts. Curve A shows the threshold value of the irradiated side of the bridge and curve B shows that of the non-irradiated side.* The threshold values of the irradiated side increased with increasing irradiation dose. The non-irradiated side first decreased for values between 20 and 30 millicurie-hours and then increased with further increase of doses. These lower values of threshold stimulus may be evidence for the occurrence of injury current from the irradiated region.

2. *Change of the action current by irradiation.* Some of the experimental data are shown in Fig. 3. At exposures higher than 20 mc h., the action current from the irradiated point became smaller in strength and in duration according to the doses administered and finally disappeared at 70 mc h.

Summary. We have made some physiological investigations of the response of a single nerve fiber to beta rays. Small exposures, up to 20 millicurie-hours at a distance of 1 mm in air with the filter shown, showed no effect.

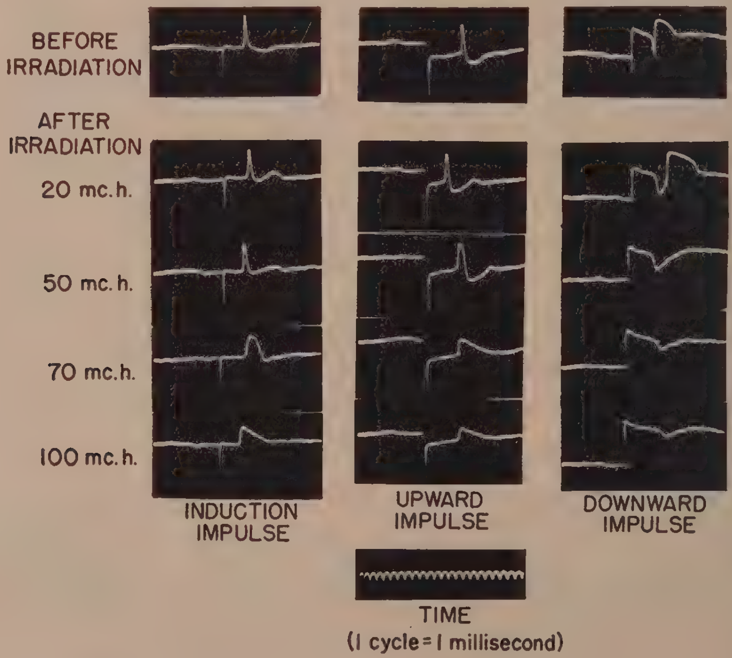

FIG. 3. Changes of action current of a single nerve fiber.

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* Each point represents an average of several experimental observations.

Moderate exposures (20-30 mc h.) evoked an injury current from the irradiated region. At higher exposures, the threshold value of stimulus increased and the action current decreased in strength and duration, and ultimately disappeared at 70 millicurie-hours. Complete breakdown occurred at the highest doses.

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A Method of Human Plasmapheresis.* (19629)

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Although there has been considerable investigation of plasmapheresis in experimental animals(1) very little information is available concerning similar studies in humans. Co Tui *et al.*(2) in the course of investigating the problem of multiple blood donations during World War II, found that the ability of man to regenerate plasma proteins was virtually unlimited provided the red blood cells were returned to the donor. In the present study 16 liters† of plasma were removed from a patient with multiple myeloma over a period of 35 days. Since the cellular elements of his blood were restored, our patient experienced no ill effects from the procedure and the erythrocyte, hemoglobin, hematocrit and serum bilirubin remained essentially unchanged.

Method. The patient was bled daily in the amount of 500 cc into a Transfuso-Vac bottle‡ (closed system). The bottle was inverted and refrigerated at 4°C for 24 hours which permitted the red cells to settle into the upper portion and neck of the donor bottle. On removal from the refrigerator care was taken not to disturb the red cell layer and a recipient set was attached to the bottle after an 18-gauge needle had been inserted into the air-vent to insure release of any remaining vacuum. The erythrocytes were permitted to flow through the recipient set into an arm vein until only the plasma was left in the bottle. Cells remaining in the lower part of the system were then flushed through with small amounts of sterile physiological salt solution. In the event that adequate sedimentation of erythrocytes is not obtained after 24 hours the blood may be centrifuged at 1000 r.p.m. for 30 minutes in the inverted donor bottle. Prolonged centrifugation at high speed is to be avoided, since it may produce excessive packing of the red blood cells. Plasma proteins were determined on the plasma layer by electrophoresis and by a modification of the Howe sodium sulfate method. The patient was placed on a nitrogen balance program in the

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† 15 additional liters of plasma removed subsequently without detriment to the patient.

‡ The transfusion apparatus is that prepared by Don Baxter & Co., Glendale, Calif.

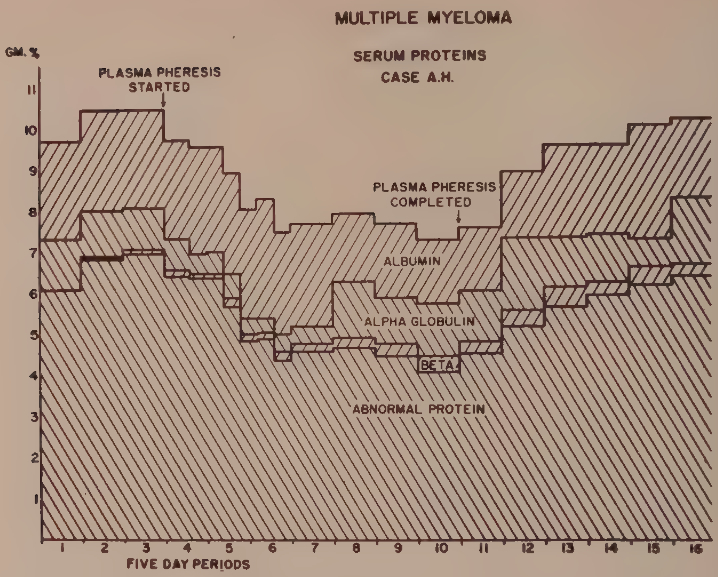

FIG. 1. Effect of removal of 16 liters of plasma on concentration of serum proteins in a patient with multiple myeloma. Note that the greatest change occurred in concentration of the abnormal protein.

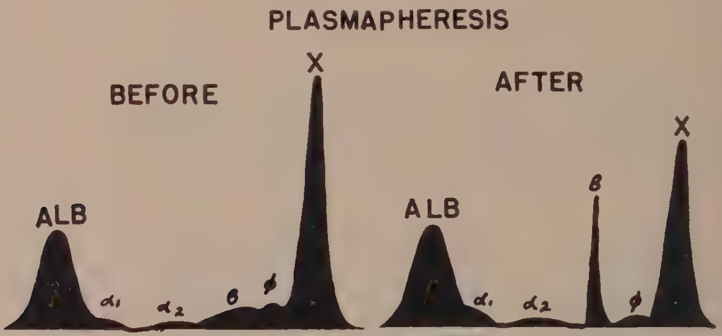

FIG. 2. Effect of removal of 16 liters of plasma on concentrations of plasma proteins as indicated by the descending electrophoretic patterns. The protein labeled "X" is an abnormal plasma protein occurring in this case of multiple myeloma. No significance is attached to the appearance of the "Beta Spike" after plasmapheresis.

conventional manner.

Results and discussion. The concentration of the abnormal plasma protein was slightly depressed whereas the concentrations of the other plasma proteins remained unchanged. (Fig. 1 and 2). On a diet composed of 85 g protein, 264 g CHO and 133 g fat (2500 calories) the patient was in slight positive nitrogen balance. When plasmapheresis was begun and the nitrogen of the protein removed by this procedure was included on the output side of the ledger, the nitrogen balance was negative by an amount approximately equivalent to the nitrogen content of the plasma removed. However, as plasmapheresis was continued, there was a gradual reduction in the excretion of urinary nitrogen which, after 20 days was equal to the plasma nitrogen. Thus it is evident that the diet ingested during this phase of the study permitted the patient to maintain nitrogen equilibrium in spite of the continuous withdrawal of 30 g of plasma pro-

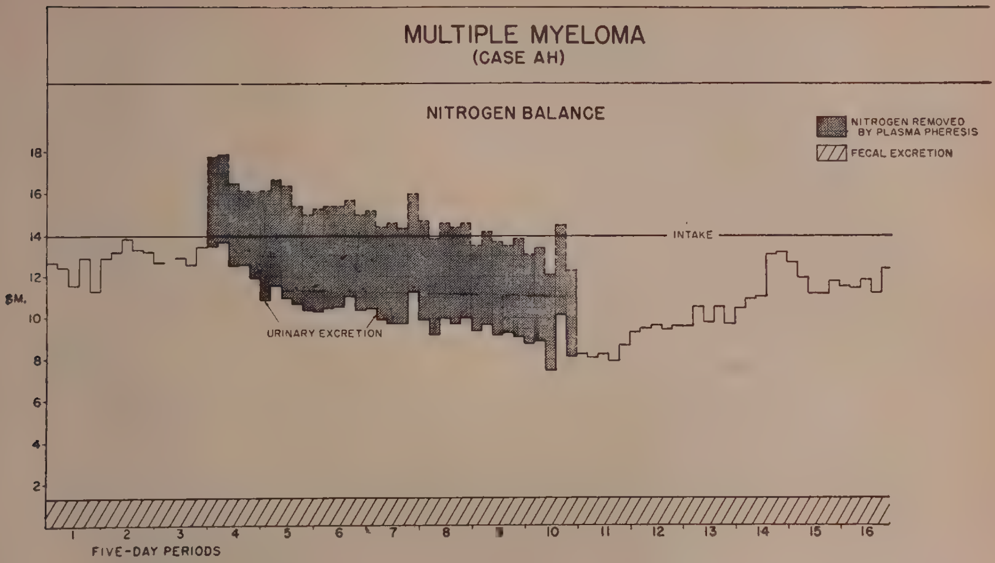

FIG. 3. Effect of plasmapheresis on nitrogen balance (see text). Dietary intake is represented as a straight line at a level of 14 g N/day. Nitrogen removed by plasmapheresis indicated by the stippled area. Cross-hatched area represents daily fecal loss of N. Daily urinary excretion of N is plotted from top of cross-hatched area to the irregular line; hence whenever this line exceeds dietary intake the patient is in negative N balance and whenever it falls below dietary line the patient is in positive N balance.

tein daily. When the plasmapheresis was discontinued at the end of 35 days the nitrogen balance became strongly positive (Fig. 3). Any initial depletion of body protein that may have occurred at the start of plasmapheresis was adequately compensated. If further study shows that the technic as outlined is a practical method for procuring large amounts of plasma from very small numbers of donors, this may well reduce the danger of transmitting viral hepatitis. In addition this suggests a method for readily obtaining large amounts of human convalescent serum.

Summary. A simple method for human plasmapheresis is described. In a single patient with multiple myeloma it has been shown that the removal of 30 g of plasma protein daily can be continued without ill effect and without substantial decrease in the concentration of plasma protein for at least five weeks. Nitrogen equilibrium was maintained when the diet contained 85 g of protein per day and 2500 calories.

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Role of Pituitary Adrenal Discharge in Antipyretic Action of Hydroxyphenyl Cinchoninic Acid (HPCA).[*] (19630)

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Adrenocorticotrophic hormone (ACTH), certain biologically active adrenocortico steroids and the common analgesic-antipyretic drugs possess certain similar pharmacologic actions(1), and it has been suggested that many actions of these latter agents may be mediated by pituitary adrenal discharge(2,3).

Recently, a derivative of cinchophen, 3-hydroxy-2-phenyl-cinchoninic acid (HPCA), has been shown to be a potent antipyretic and to cause pituitary adrenal stimulation(4,5). Since it has been reported that ACTH and cortisone possess antipyretic properties(6-9), it seemed of interest to know if HPCA is antipyretic by virtue of its ability to cause pituitary adrenal discharge. If HPCA reduces fever indirectly by causing adrenal hormone release, the time-course and intensity of the antipyresis observed following administration of the pituitary adrenal hormones and the antipyretic drug should be analogous, and a single dose of ACTH or cortisone might be expected to produce an immediate reduction in fever, similar to that reported for HPCA.

Methods. The effect of single doses of ACTH, cortisone and HPCA on the febrile response of dogs to purified bacterial pyrogen has been studied. The pyrogen,[†] a purified extract of *Pseudomonas aeruginosa*, was given intravenously in a dose of 40 μg/kg. The ACTH[‡] was given intravenously in a dose equivalent to 50 mg/animal of the La-1-A standard. Cortisone acetate was given intramuscularly, 25 mg/animal. The HPCA[‡] was dissolved in 2.7% Na_2CO_3 and given intravenously in a dose of 40 mg/kg. Twelve healthy adult 8-15 kg dogs were used in each experiment. On the first day 6 dogs were given the antipyretic drug 20 minutes prior to injection of pyrogen; the other 6 dogs were given pyrogen alone and served as controls. One week later the procedure was reversed and the 6 dogs previously pretreated now served as controls and vice versa. Such a cross-over technic was used in an effort to randomize the effects of tolerance that are known to occur to the febrile response to pyrogen(10). Two such experiments were performed using ACTH, and one each using cortisone and HPCA. One dog failed to receive the pyrogen in one ACTH experiment and there are only 11 dogs represented in that experiment. Rectal temperatures were determined every 45 minutes for 90 minutes prior to, and 360 minutes following injection of pyrogen. All temperatures are recorded in Fahrenheit degrees, and are increments from the mean of the prepyrogen temperature determinations.

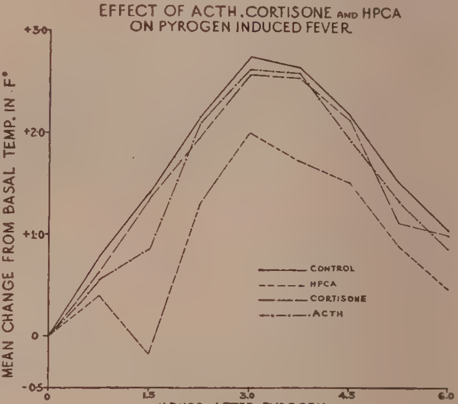

FIG. 1.

Results. Fig. 1 presents the mean febrile responses for the pooled controls, and following each of the drugs tested. As may be seen, only HPCA caused appreciable reduction in the febrile response. The mean increments during the 6-hour period, and the maximum increments have been analyzed statistically. Each dog served as its own control in each

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[*] This investigation was supported by research grants from the National Institutes of Health, U. S. Public Health Service, and Baxter Laboratories. Morton Grove, Ill.

[†] Kindly supplied by Dr. L. G. Ginger of Baxter Laboratories. This preparation is marketed under the name of "Pyromen."

[‡] Kindly supplied by Dr. John Litchfield, American Cyanamid Co., Stamford, Conn.

TABLE I. Effect of ACTH, Cortisone and HPCA on Pyrogen-Induced Fever.

	ACTH (23 dogs)		Cortisone (12 dogs)		HPCA (12 dogs)	
	Control	Pretreat	Control	Pretreat	Control	Pretreat
Prepyrogen temp.	101.80±.13	101.81±.15	101.83±.21	101.82±.21	101.73±.18	101.63±.17
Mean increment	2.04±.20	1.70±.16	1.67±.22	1.77±.22	1.85±.20	1.03±.14
Mean diff.	−.336±.21		+.099±.07		−.828±.21	
Significance	0		0		High	
Max increment	3.10±.18	3.85±.22	2.75±.29	2.83±.26	2.87±.24	2.02±.20
Mean diff.	−.248±.23		+.083±.15		−.842±.31	
Significance	0		0		High	

All values are in Fahrenheit degrees and are followed by their stand. errors.

experiment and paired comparisons were used to calculate the t ratios. These data are presented in Table I. It is apparent that ACTH and cortisone, in contrast to HPCA, failed to reduce either the maximum increment or the mean 6-hour increment following pyrogen. Since neither the average nor the maximum fever was affected by ACTH or cortisone, it seems unlikely that pituitary adrenal discharge caused by HPCA is an important factor in the antipyretic action of this drug.

The failure, in these experiments, of ACTH and cortisone to reduce fever prompted reexamination of the evidence presented to show that these agents are antipyretic. In most studies, in which an antipyretic action was demonstrated, ACTH or cortisone was given in multiple doses bginning about 24-36 hours before testing the animals' response to a standard pyrogen(6-8). In only one study (9) was there reported an immediate antipyretic action; graphs were presented showing a delayed and diminished pyrogen-induced fever in 3 rabbits immediately after a single injection of 2-3 units of ACTH. Defervescence also appeared to be accelerated in 4 rabbits when ACTH was given at the peak of the fever. However, clinical reports indicate that there is, in febrile patients, a time delay of about 24 hours between the inception of ACTH or cortisone therapy and the resultant antipyresis(11,12). It has been suggested, in fact, that small doses of ACTH or cortisone administered only 24 hours prior to pyrogen may result in augmentation of the febrile response(8). It appears, therefore, that ACTH or cortisone therapy must be initiated about 24 hours before the antipyretic action of these agents becomes manifest and, further, that the prompt antipyresis subsequent to the administration of HPCA is not to be attributed to intermediation of the pituitary adrenal system.

Conclusions. ACTH and cortisone, under the conditions of this experiment, were without significant effect on the febrile response of dogs to pyrogen. In contrast, hydroxyphenylcinchoninic acid produced a significant reduction in the pyrogen-induced fever. It is concluded that HPCA, and perhaps similar common antipyretic drugs, do not cause a reduction in fever by virtue of their ability to cause pituitary adrenal stimulation.

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Adaptation of Hypophysectomized Rat to Fasting. (19631)

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Since the hypophysectomized rat in several instances had been found to a tendency to develop hypoglycemia during fasting(1-4), it seemed likely that it would succumb readily to fasting. The present study was undertaken because it had been observed that hypophysectomized rats whose nutrition had been maintained by stomach tube feeding seemed to survive fasting rather better than had been expected. Inasmuch as it appeared that the maintenance of nutrition by such feeding was the explanation, the study was arranged to check on this possibility.

Procedure. Male albino rats obtained from Sprague-Dawley and of the same age were sorted into groups of similar average weight. The animals were hypophysectomized or sham operated by a parapharyngeal approach. The pituitary was exposed but not otherwise disturbed in those sham operated. At the end of the experiment, the completeness of the removal of the pituitary was checked by serially sectioning a block of tissue including the base of the skull and the overlying brain. Every tenth section was mounted and stained. The sections were cut 10 μ thick. In all the hypophysectomized animals from which data are reported there was no trace of pituitary within the dural capsule which normally contains it, nor were any implants found along the operative path. The stalk lying between the infundibulum and dural capsule always remained intact, for care was taken at the time of the operation not to rupture the dorsal layer of the capsule. The animals were allowed to fast for the 2 days following the operation. They were then given a liquid diet in which 20% of the calories were from protein and 40% each from carbohydrate and fat. Those fed by stomach tube were given 2 or 3 feedings per day of sufficient quantities to induce a gain in weight(5). The amounts given the hypophysectomized rats and their sham operated controls were identical. After 19 days of feeding, the animals were allowed to fast until they died. During the period of fasting they were kept in a laboratory temperature of approximately 25-27°C. They were housed in cages with screen bottoms, but because it was expected that at least those hypophysectomized rats which had been allowed to feed *ad libitum* would promptly succumb to fasting and thus terminate the experiment, several animals were placed in each cage. Consequently their oral intake may have included more hair and debris such as rats get in grooming each other as well as more feces due to coprophagia than if they had been caged singly. In addition a few grams of tissues were eaten from each of 2 animals, one dying on the 11th and the other on the 12th day of fasting. The fasting conditions, therefore, were not absolute; but it seems reasonable to describe them as severe. Furthermore, there is no reason to suspect that they favored the hypophysectomized animals as compared with their controls. Tap water was offered *ad libitum.* Blood sugar was determined by a modification of the Folin-Malmros ferricyanide method on samples taken from the tip of the tail(6).

The data from 3 force fed hypophysectomized rats dying on the 2nd, 3rd and 11th day of fasting and from one hypophysectomized rat fed *ad libitum* dying on the 12th day of fasting were discarded. The animals dying on the 2nd and 3rd days were found to have multiple pulmonary abscesses at postmortem.

Results and discussion. The average body weight and blood sugar concentration of the groups are shown in Fig. 1. The numerical data at selected points on these curves are given in Table I together with the data for the postmortem weight of the body, liver and testes and the number of days the animals survived fasting. It appears that a number of hypophysectomized rats which had been allowed to feed *ad libitum* as well as many of those which had been force fed survived

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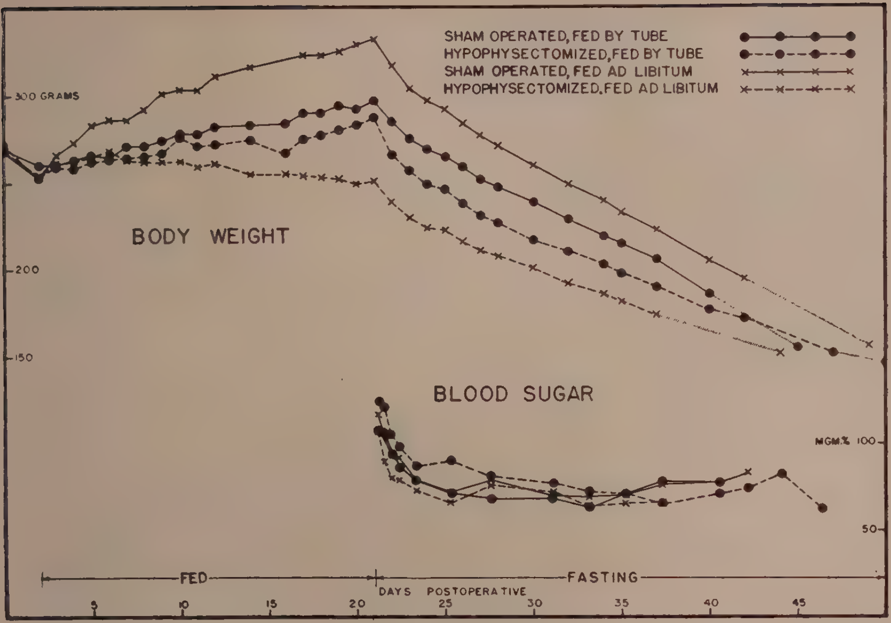

FIG. 1. Avg body wt and blood sugar concentrations. The final points on the body wt curves show avg body wt and avg day of death for respective groups.

fasting about as long as their controls. It further seems that these hypophysectomized animals which survived fasting well on the average were able to maintain their blood sugars at values not differing greatly from those of their controls. At times the hypophysectomized rats fed *ad libitum* did have a lower average blood sugar concentration than their controls in agreement with the findings in previous studies. When the averages over a longer period are considered, however, the differences are not impressive. It also may be noted that at death from inanition the controls had smaller livers and larger testes. While the hypophysectomized animals fed *ad libitum* apparently gained less weight during the period of feeding, yet they seem to have compensated for this deficit by consuming their weight more slowly during fasting. It may be pertinent that the hypophysectomized animal tends to have a higher percentage of body fat and that smaller weight changes are involved in either storing or consuming a calory in the form of fat rather than protein(7).

It is evident that the prior maintenance of its nutrition by stomach tube feeding is not essential for the fasting hypophysectomized rat to maintain its blood sugar concentration above that at which the symptoms of hypoglycemia appear and to survive for about as long as its control.

Evidence has been offered that suggests that the hypophysectomized rat suffers defects both in removing an excess of sugar from its blood and in supplying sugar to prevent or overcome low blood concentrations(9,10). The findings of the present study do not indicate that such defects are absent, but merely that if they are present they do not necessarily lead to hypoglycemia and an early death during fasting.

Summary. It was found that in a group of hypophysectomized rats allowed to feed *ad libitum* as well as in a group force fed by stomach tube some animals subsequently survived fasting about at long as their non-hypophysectomized controls. These hypophysectomized animals which survived fasting well also maintained blood sugar concentrations

TABLE I. Numerical Data, Averages and Ranges (in Parentheses) for Groups.

	Sham operated, fed by stomach tube	Hypophysectomized, fed by stomach tube	Sham operated, fed *ad lib.*	Hypophysectomized, fed *ad lib.*
No. of animals	7	5	7	9
Body wt, g				
Day of operation	270 (258–283)	272 (262–287)	268 (261–282)	269 (260–285)
Beginning of fast	297 (282–314)	287 (280–293)	332 (298–353)	251 (237–266)
16th day of fast	209 (193–215)	190 (182–197)	223 (199–234)	174 (161–187)
Day animal died	155 (148–172)	146 (138–153)	156 (150–174)	152 (135–170)
Blood sugar, mg %				
Beginning of fast	108 (94–116)	124 (101–147)	117 (101–134)	107 (93–114)
2nd day " "	78 (72–84)	87 (78–96)	79 (71–95)	73 (62–84)
16th " " "	78 (65–102)	65 (46–72)	76 (64–98)	66 (54–84)
Survival after beginning of fast in days	24 (19–28)	29 (27–31)	28 (23–31)	23 (18–29)
Organ wt at death, g				
Testes	1.96 (1.76–2.20)	.35 (.28– .42)	1.93 (1.60–2.38)	.37 (.29– .44)
Liver	2.1 (1.8 –2.3)	3.3 (3 –3.6)	2.2 (1.9 –2.6)	3.6 (3 –5)

during fasting which were comparable to those of their controls.

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Spermicidal Effect of Certain Physiologically Active Substances. (19632)

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A great number of compounds have been tested to determine their action on spermatozoa. Among those which have been found to be toxic are alkyl hydrocupreidine derivatives(1), metabolites such as indole, maleate, and glyceraldehyde(2), and long chain compounds such as sulfo-dioctylsuccinate(3). Ketone reagents have also been reported to be toxic(4), as have known enzyme inhibitors like fluoride and iodoacetate(5). Heavy metal salts, like phenylmercuric acetate(6,7), wetting agents, such as Duponol(8), and quinones(9) have been found to have toxic properties.

It is apparent that a large number of compounds of widely varying chemical structure have in common the property of toxicity toward spermatozoa. For this reason the possibility is relatively high that a given compound may have some spermatocidal effect. The ever increasing number and variety of chemical compounds in clinical use suggested that it might be of value to determine whether such substances would have a deleterious effect on spermatozoa, since this might affect their utility as therapeutic agents. In addition it was felt that the possibility of discovering one or more spermatocidal agents in types of compounds not hitherto extensively studied warranted the investigation reported here.

Experimental. Spermatocidal activity was determined by a modification of the method of Lardy and Phillips(10). Rat sperm were used. The rats were killed with ether and the testes removed immediately and cut in half. Testicular contents were removed with a platinum loop and flushed in 0.08 ml of Locke-Ringer solution on a glass slide. A cover glass was then placed over the solution and the slide was examined microscopically at 10-minute intervals. One testicle of each rat served as the control; the other testicle was treated with Locke-Ringer solution in which was dissolved the compound being tested. Under these conditions the control sperm were usually motile for a period of 30 minutes. Any shortening of this period was taken as indicating spermatocidal activity by the compound being tested. Compounds were run at serial dilutions. The highest concentration used was 1.0 mg/ml. Several series of compounds were tested. These included a group of wetting agents and a number of flavonoid compounds; the latter were run because they are known to form quinones in biological media(11), and quinones are known to have spermatocidal properties(9). Also investigated were a number of surface-active agents and a group of metabolite analogs. Particular attention was paid within this last group to analogs of pteroylglutamic acid, purines and pyrimidines, because of the high concentration of nucleic acids known to occur in spermatozoa. Finally a number of therapeutic agents were tested. An attempt was made in this group to cover as wide a range of therapy as possible, so that the group includes representatives of such pharmaceutical classes as antihistamines, antibiotics, etc. A number of standard spermicides were tested to provide a basis for comparison.

Results and discussion. Standard spermicides tested were lactic acid, oxyquinoline sulfate and phenylmercuric nitrate. Their minimum effective concentrations (M.E.C.) were, respectively, 1.0, 0.1 and 0.01 mg/cc.

Members of each of the other groups tested were found to show activity. In the flavonoid compounds, activity appeared to depend on ability for quinone formation. The most active compounds of the group were quercetin, dihydroquercetin, rufigallic acid and 3,3′,4,4′ tetrahydroxy chalcone, all of which had an M.E.C. of 0.1 mg/cc. A reduction in the number of possible quinone-forming groups reduced the activity; thus rutin and 3,4-dihydroxy chalcone had an M.E.C. of 1.0 mg/cc. Compounds which structurally cannot form quinones, such as hesperidin methyl chalcone and esculin, were inactive.

The results obtained with surface-active agents were not consistent. Although all the materials tested function in the same manner, some had a spermatocidal effect and some did not. A number of Spans and Tweens tested, for example, were inactive. Three samples of Triton had M.E.C.'s ranging from 1.0-0.1 mg/cc. This behavior would lead to the conclusion that spermicidal action by these agents is a function of their chemical structure rather than of their surface-active properties.

The activity of the metabolite analogs tested appears to be capable of rough classification. No appreciable effect was shown by analogs of phenylalanine, threonine, glycine, alanine or tyrosine. Analogs of thiamine, pantothenic acid and pyridoxine were likewise inactive. 2-Methoxy-1,4-naphthoquinone was active, having an M.E.C. of 0.1 mg/cc, but whether this is a manifestation of anti-vitamin properties is doubtful, since the quinone structure as such is known to have activity.

Pteroylglutamic acid analogs, however, showed spermatocidal activity as a group. 4-(((2-amino-4-hydroxy-6 pteridyl)-ethyl)-amino)-benzoic acid was particularly active among these compounds, giving an M.E.C. of 0.1 mg/cc. Activity at 1.0 mg/cc was shown by 12 other analogs tested. These represented structural modifications including replacement of the 6-pteridyl grouping with a 7-pteridyl, substitution of an aspartic acid grouping for the glutamic, inclusion of a methyl group on the pteridine nucleus, and replacement of the 2-amino group with a hydroxyl. The activity shown by these compounds appeared to depend on the pteridine nucleus, since replacement of this with a quinazoline grouping caused an inactivation. There is furthermore a correlation, although not a perfect one, between the anti-folic acid activity of these compounds and their spermatocidal effect(12). The possibility is thus raised that the spermatocidal effect of folic acid antagonists is due to a displacement of folic acid in the sperm cell. The importance of nucleic acids in the sperm cell and the function of folic acid in purine and pyrimidine synthesis lend more interest to this possibility. The purine and pyrimidine analogs tested were generally ineffective, although a few, particularly 2,4,5-triamino-6-hydroxy-pyrimidine, had appreciable activity. This had an M.E.C. of 0.1 mg/cc. Hitherto studies on the effect of inhibitors on spermatazoan metabolism have been concerned mainly with glycolytic mechanisms; the results discussed above suggest the utility of further study on purine and pyrimidine metabolism.

The group of therapeutic substances tested included representatives of the classes of diuretics, antihistamines, analgesics, and adrenolytics. The results obtained presented no coherent picture, and it would appear that spermatocidal activity in this group is a function of chemical structure rather than of therapeutic effect.

It is worthy of note that, among a number of compounds tested which do not fit into any of the above classifications, coumalic acid was particularly effective, having an M.E.C. of 0.01 mg/cc.

Summary. A number of compounds of different chemical structure and physiological effects were tested to determine their spermatocidal properties. Several were found to be active, including a group of pteroylglutamic acid analogs.

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Renotrophic-Androgenic Properties of Orally Administered Androgens.* (19633)

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Previous studies(1,2) have indicated that among the androgenic steroids the 17β-hydroxyl group is essential for maximum renotrophic activity and the 3-ketone group for androgenic activity in the castrated mouse. On the basis of these observations an attempt(3) was made to obtain further separation of these two properties of the androgens by studying compounds with a functional group in only one of these two positions. Such compounds administered parenterally, however, proved to be very insoluble in the tissue fluids. It seemed that another route of administration might prove more efficacious. Therefore, since one of these compounds, 17-methylandrostan-17β-ol, was still available, it was studied by oral administration and its properties compared with several other available 17-methyl androgens administered by the same route. At the same time a few of the non-methylated steroids were studied, for although it is assumed that these compounds are inactivated by the liver, there is ample evidence(4,5,6,7) which indicates that androgens can be converted to more potent androgens by incubation with liver slices or homogenates.

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Experimental. Male white mice, from the Maple Grove Rabbitry, Springfield, Mo., were castrated at 17 to 19 g body weight. The mice were kept 2 in a glass jar containing a false screen bottom in a room air-conditioned at 24-26°C. They were fed and weighed at the same time on Monday, Wednesday and Friday of each week. The amount eaten was determined by weighing the food at the beginning and end of each interval. The regular diet consisted of casein 16.7, sucrose 61.2, hydrogenated vegetable oil 7.4, yeast (Fleischmann's 2019) 9.2, Celluflour 1.8, and Wesson's salt mixture 3.7. At each feeding 2 drops of Patch's cod liver oil and 1 drop of 34% tocopherol[‡] concentrate from wheat germ oil diluted 10-fold with Wesson oil were mixed with the food.

The androgens[§] were dissolved in 10 to 15 ml ethyl alcohol, and were mixed thoroughly with the food by rotating the glass container. The amount necessary for the experimental period was prepared at one time and kept at 3 to 5°C. This mixture was substituted for the regular diet 30 days after castration and the mice were autopsied 30 days later. The organs were weighed on Roller-Smith torsion balances. *Results.* The amount of food eaten by the mice in the various groups was roughly identical.

As expected, 17-methyltestosterone proved to be the most effective steroid. The steroid of greatest interest, however, is 17-methylandrostan-17β-ol which has no group at the 3 position. At the lower dose it demonstrated a renotrophic potency roughly equivalent to that of 17-methyltestosterone and at 0.8 mg[‖] /day was almost as effective as 17-methyltestosterone at 2.0 mg/day. Furthermore, in both instances the androgenic activity of 17-methylandrostan-17β-ol was much less than that of 17-methyltestosterone.

The non-methylated steroids were all effective except Δ^4-androstene-3,17-dione at the lower dose level. Testosterone and androstane-3α,17β-diol, however, were not as potent as their 17-methyl derivatives.

An increase in the dose of the androgens resulted in a further increase in androgenic activity without a concomitant increase in renotrophic activity. This phenomenon is not peculiar to the oral administration of the androgens, but is also very evident after parenteral administration(2,7).

The thymus was decreased in size by the various androgens in roughly reverse proportion to the increase in size of the seminal vesicles and prostates.

Discussion. A comparison of the results obtained by oral administration of androgens with the previous results(1,2) obtained after subcutaneous implantation of pellets of these steroids indicates that the oral route is much less efficient. But those steroids which are only slightly soluble in tissue fluids(1,2,3) show a much greater comparative potency when administered by mouth.

This is specially true for 17-methylandrostan-17β-ol, which, though ineffective by parenteral administration(3), proved not only to be highly active by mouth but also showed preferential renotrophic activity. This property of this compound is of special interest for, by removal of an active group from the 3 position, a further reduction in androgenic activity without impairment of the renotrophic activity has been obtained. Thus, the relationship of the chemical group at the 3 position to androgenic activity can be extended to give the following sequence: $C = O > \alpha\ OH > \beta\ OH >$ no group.

Summary. Orally administered non-methylated as well as methylated androgens are able to stimulate an increase in size of the kidney and the seminal vesicles and prostates and decrease the thymus of castrated mice. The

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[‡] Provided by Distillation Products Inc. through the courtesy of Dr. P. L. Harris.

[§] The androgens were generously made available by Ciba Pharmaceutical Products, Inc.

[‖] The amount of this steroid available was not sufficient to permit an experiment at the 0.5 mg/g food (2 mg/day) level.

TABLE I. The Renotrophic and Androgenic Effect of Orally Administered Androgens.*

		Body wt		Food				Incr.†
	Mice No.	Initial, g	Change, g	intake, g/day	Thymus, mg	Kidney, mg	SVPr, mg	K/SVPr, mg/mg
	.1 mg steroid/g food, 30 days							
Control (castrated)	8	31.2	4.4	3.7	64±5	355±13	16±1	—
17-Methyltestosterone	7	28.6	5.1	4.2	36±6	478±36	112±8	1.28
17-Methyl-Δ^5-androstene-3β,17β-diol	7	30.4	4.7	4	38±4	452±27	42±6	3.73
17-Methylandrostan-17β-ol	4	29.9	3.7	3.8	34±9	446±29	40±7	3.80
Testosterone	6	29.4	5.5	4.1	52±4	426±26	51±10	2.32
17-Methylandrostane-3α,17β-diol	6	28.6	5.6	3.9	34±4	412±21	57±9	1.43
3β,17β-diol	5	29.6	5.5	3.8	39±5	399±20	34±8	2.45
17-Methylandrostan-17β-ol,3-one	4	27.2	3.3	3.7	29±8	395±23	59±9	.93
Androstane-3α,17β-diol	6	30.2	4.7	3.9	50±5	394±20	25±4	4.33
Δ^4-Androstene-3,17-dione	6	30.4	5.2	3.8	52±6	379±27	17±2	—
	.5 mg steroid/g food, 30 days							
Control (castrated)	8	29.7	4.1	4	47±5	367±18	15±3	—
17-Methyltestosterone	6	31.4	4.1	4	6±1	565±36	307±26	.68
17-Methylandrostan-17β-ol‡	4	31.4	7	3.9	37±5	523±26	100±16	1.84
17-Methylandrostane-3α,17β-diol	6	30.8	4.5	4.3	8±3	514±14	232±27	.68
17-Methylandrostan-17β-ol,3-one	4	30.2	4.1	4.3	4±3	488±29	226±26	.45
17-Methyl-Δ^5-androstene-3β,17β-diol	6	31.7	6	3.9	23±8	482±16	189±48	.66
Androstane-3α,17β-diol	7	31.8	7.3	4	35±3	455±32	90±18	1.49
17-Methylandrostane-3β,17β-diol	8	31.4	3.5	4	27±5	451±15	137±20	.70
Testosterone	6	31	4.8	3.9	30±8	420±26	160±19	.36
Δ^4-Androstene-3,17-dione	6	31.3	6.3	4	44±6	398±23	36±6	1.43

* The values for the organ weights are given with the stand. error of the mean $\dfrac{\sqrt{\dfrac{X^2}{N} - \bar{X}^2}}{\sqrt{N-1}}$.

† Increase in kidney wt divided by increase in seminal vesicles and prostates wt.

‡ This steroid was at only .2 mg/g of food because of unavailability of further material.

absence of a functional group in the 3 position, *e.g.*, 17-methylandrostan-17β-ol resulted in a marked decrease in androgenic, but not renotrophic, activity.

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Fetal X-Irradiation and Fertility.* (19634)

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It is important to determine, with experimental animals, the effect on subsequent fertility of high but sub-lethal exposures of the fetus. For this study the Swiss mouse was used, the pregnant female receiving chronic exposures for 6 days prior to delivery. The offspring were then tested at 6 months (the height of normal reproductive activity) for fertility. There have been numerous reports of the effect on the mammalian gonad of di-

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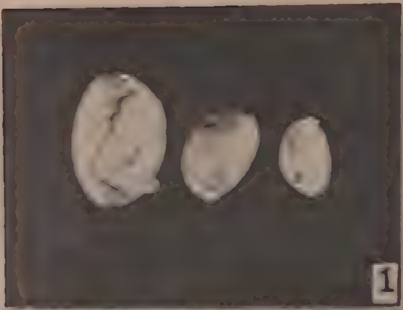
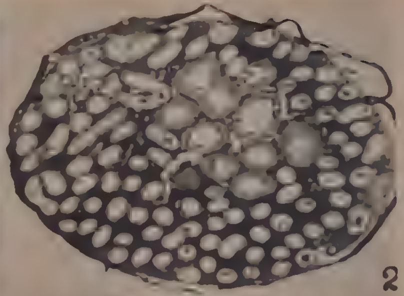
rect x-irradiation (Bergonie and Tribondeau (1), Eschenbrenner *et al.*(2-5),) and in the earlier reports the primary spermatocytes were regarded as particularly radiosensitive but Regaud and Blanc(6), Schintz and Slotopolsky(7), Wigoder(8), Metz(9), and Lacassagne and Gricouroff(10), claim that the spermatogonia are the more radiosensitive. In general, the ovary was regarded as less sensitive (Blass) (11) until recent studies, possibly for the reason that the histological changes in the ovary are less obvious. Nevertheless, histological and physiological changes in the adult ovary have been described following direct x-irradiation (Regaud and Lacassagne(12), Brambell *et al.*(13), Goldstein (14), Murray(15), Rock *et al.*(16), Peck *et al.*(17), van Eck-Vermande(18), Halberstaedter and Ickowica(19)). Brambell and Parkes(20), found a compensatory hypertrophy of the untreated ovary in unilateral x-ray sterilization and recently Furth(21) and Gardner(22), described the induction of ovarian tumors by x-rays. X-irradiation has been used to produce temporary or permanent sterility in experimental animals and in human subjects (Dantchakoff and Lacassagne(23), Glucksmann(24), Mason and Shaver(25), Shaver and Mason(26), Russell(27), Kaplan (28), and Metcalf and Inda(29)). Kaplan claims that x-irradiation sterilization of the human female need not be permanent, and that females may be temporarily sterilized and yet subsequently become pregnant and deliver what he terms as normal offspring (Kaplan)(28). He did not consider the genotypic implications as does Muller(30). Other studies on offspring of x-irradiated parents have been made (Doderlein(31), Murphy (32), Snell and Ames(33), Henson(34), and Nicolle(35)). Curiously, in the face of these studies, x-irradiation has been used in a few instances to promote ovulation (Haman)(36).

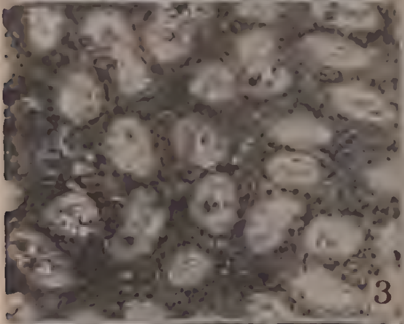
In this study it was decided to give the mouse fetus 6 daily exposures, each equivalent to a substantial fluoroscopic examination (50 r) and totalling a dose which was known to be tolerable (300 r). It was further decided to test the mice for fertility at one time only, namely 6 months, in order to avoid the possible stimulation of the gonads by prior reproductive activity. The results of this study are presented below.

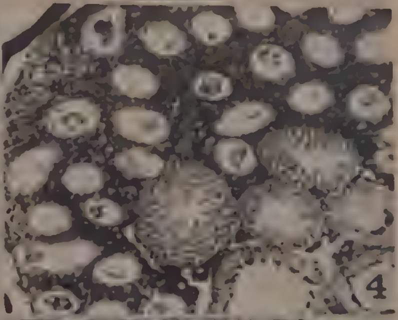
Materials and method. Ten pregnant Swiss mice were given whole body x-irradiation to 50 r for each of 6 consecutive days beginning on the 14th day of gestation. (50 r is comparable to an extended fluoroscopic examination). This meant a total accumulated exposure of 300 r before parturition, a dose which was known to have a slight stunting effect on the offspring if given at one time late in pregnancy. By fractionating the dose in this manner apparently normal litters were produced without any visible effects on size or viability. Ten similarly pregnant female Swiss mice were maintained as controls. At the normal time of weaning (4 weeks of age) the males and females were separated into groups of 5 per cage, all similarly treated, and were maintained for 6 months on the standard laboratory diet. At the end of the 6 months period, at a time when one expects a normal mouse to be at the height of its reproductive activity, all surviving mice were tested for fertility. A total of 45 experimental (x-irradiated) and 58 control offspring were so segregated at weaning and maintained. The test for fertility for each male was made against 2 normal females, each of which was known to have had a previous litter by a normal male. The test for the female was exposure to 2 males, at successive 5-day intervals, each male having been previously used to produce a normal litter with a normal female. Since the mouse has a 4½ day estrous cycle, 2 males successively available to the female over a 10-day period should certainly have tested its fertility. The x-irradiation facilities were the Westinghouse Therapy machine of this department, regulated to 210 kv and 15 a, at a target distance of 50 cm and output of 96.2 r per minute with filtration of 0.28 mm Cu and 0.50 mm A1.

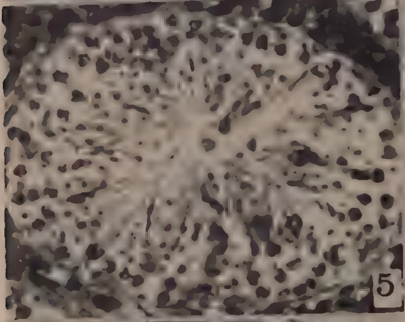
Experimental data and observations. All mice, controls and experimental, maintained for 6 months will show some mortality. All x-irradiated pregnant females were thereby made subsequently sterile. Of the 58 control offspring 51 or 88% survived. Of the 45 x-irradiated offspring 40 or 89% survived. Irradiation under these conditions did not

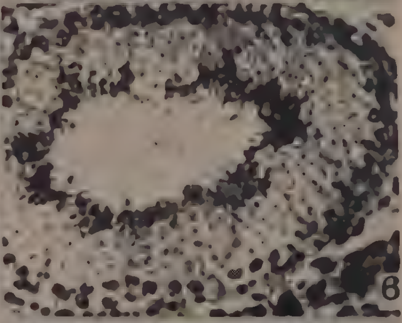


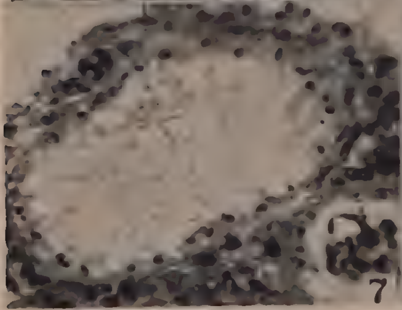












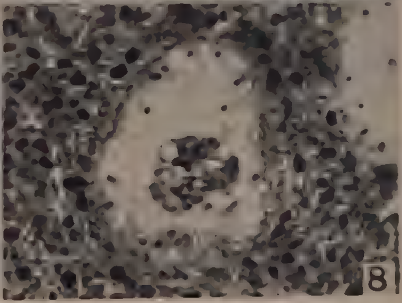


FIG. 1. Whole testes excised and photographed fresh. Control on the left, x-irradiated but fertile in center, and x-irradiated but sterile to the right.

FIG. 2. Central section of sterile testes showing about 10% of the tubules containing maturation stages. Note amount of interstitial tissue in sterile and non-sterile portions of the section. Hyperplasia of the interstitial tissue is absolute but the impression is accentuated because of large size of the non-sterile tubules as compared with those that are completely sterile.

FIG. 3. Completely sterile portion of x-irradiated testes.

FIG. 4. Enlarged view of sterile and non-sterile portions of Fig. 2 testes.

FIG. 5 and 6. Seminiferous tubules containing maturation stages in x-irradiated but fertile testes.

FIG. 7 and 8. Completely sterile seminiferous tubules in x-irradiated testes showing intact Sertoli cells and interstitial tissue, the latter being increased in amount. In Fig. 8 note accumulation within the lumen of a few loosened Sertoli cells and probably some abortive early maturation stages.

alter survival. Of the controls, 27 out of 30 males and 24 out of 28 females survived while among the experimentals all of the males and 13 of the 18 females survived. Male survival among the x-irradiated offspring was therefore somewhat better than female survival, but this probably has no significance because of the small numbers. The surviving control mice were slightly heavier than the x-irradiated experimentals.

The fertility tests resulted as follows:

Controls:
51—all fertile

X-irradiated:

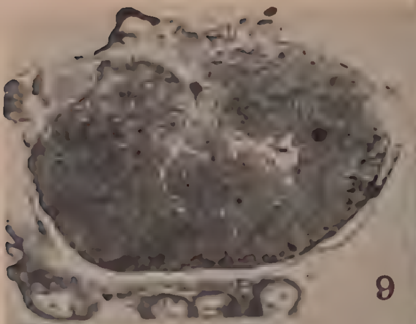
Males	27	Females	13	
Fertile	5	Fertile	9	(2 with abnormal litters)
Sterile	22	Sterile	4	

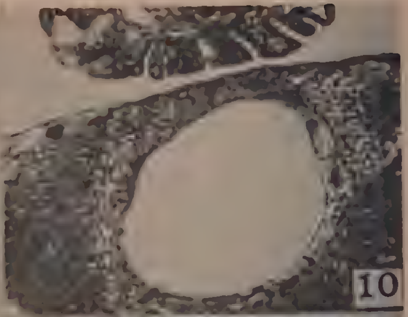
The gonads of all experimental animals and 12 controls (6 males and 6 females) were excised and fixed for histological analysis.

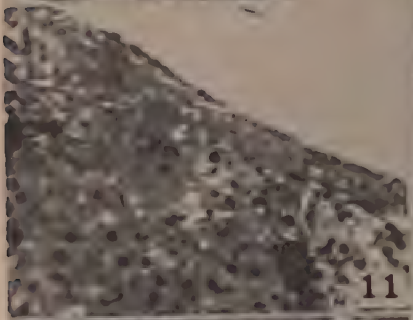
It was noted immediately that there was a wide range of sizes among the testes of the x-irradiated males (Fig. 1), but no such size variable was seen in the ovaries. The smallest testes were invariably found in the sterile x-irradiated males (Fig. 3, 7, 8) while the fertile but x-irradiated males had testes of intermediate size between those that were sterile and the controls (Fig. 1). There was always some size reduction in all x-irradiated testes. It was estimated that the volume of the completely sterile testes was roughly 25% that of the controls, with the maximum length being reduced from some 10 mm to 3 mm (Fig. 1). The size variation among the testes of x-irradiated males was correlated with the degree of histological integrity, the larger ones being the more normal. One could say that if the testis was reduced to below 40% of its original (normal) size, it would be sterile even though it could produce some spermatozoa (Fig. 2). If the testis is somewhat above 40% the volume of the control testis, it would, in all probability, be able to produce a sufficient number of viable spermatozoa to be considered as fertile (Fig. 5 and 6).

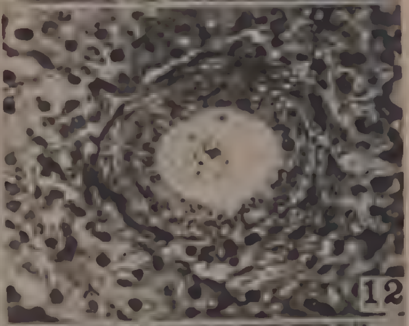
Histological analysis. Control testis of the mouse at 6 months is perfectly normal for the mammalian gonad at the height of its activity. It contains approximately 100 seminiferous tubules in any central cross section, each showing maturation stages and most of them showing all stages from spermatogonia to mature spermatozoa. The tubules are approximately of the same size in cross section, and are clearly delineated from each other so that in fixed material they appear to be separated by spaces. This is, in part, because of the relative sparsity of the interstitial tissue in the controls. In the testes of mice found to be sterile 6 months after x-irradiation *in utero,* never more than 10% of the seminiferous tubules could in any way be compared with the controls with respect to cellular constituents (Fig. 2 and 4). When one counts the completely sterile tubules (*i.e.,* devoid of maturation stages of any sort) and compares them with the tubules which contained recognizable maturation stages, one gets a range of ratios from 2/103 to 10/91 or approximately 2 to 10%. The few normal appearing tubules are generally bunched together, often toward the center of the testes (Fig. 4).

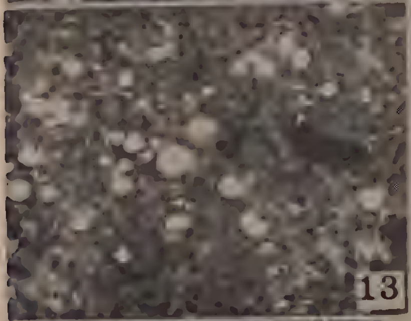
Most of the completely sterile tubules are devoid of all maturation stages but do retain their Sertoli cells in which the nuclei appear as (generally 3) beads linearly joined (Fig. 3, 7, and 8). The cytoplasm is clear and the cell is rounded rather than elongate as in the control tubules. A few of the tubules in the sterile testes may be completely devoid of the Sertoli cells and contain only a fibrous debris. There is sometimes a coagulum within the lumen of some of these sterile tubules, the coagulum consisting of some loosened Sertoli cells and unrecognizable cells which may be abortive maturation stages (Fig. 8). The

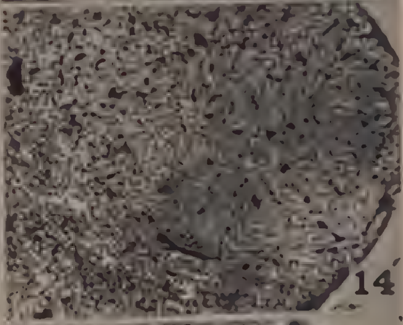


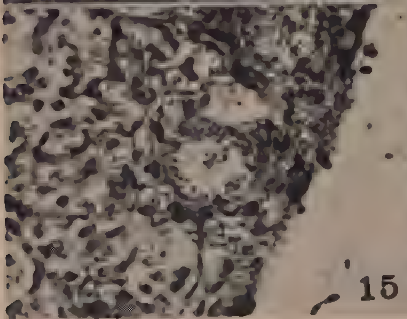












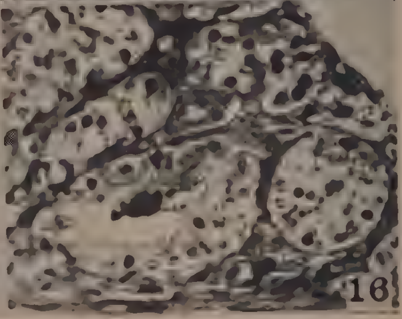


FIG. 9. Low power view of central section through completely sterile ovary.

FIG. 10. Enlarged view of cyst found in sterile ovary.

FIG. 11. Marginal germinal epithelium of x-irradiated ovary appears normal but the underlying tissue does not contain germinal nests.

FIG. 12. Partially developed but abnormal follicle containing ovum in x-irradiated mouse from whom 2 aborted and abnormal fetuses were delivered following mating with normal male.

FIG. 13. Fatty degeneration in x-irradiated ovarian stroma.

FIG. 14. Ill-defined corpus luteum in ovary from which abnormal fetuses were delivered.

FIG. 15. Anovular rings and irregular germinal epithelium on x-irradiated ovary, completely sterile.

FIG. 16. Germinal nests which do not develop further in x-irradiated ovary, incompletely sterile.

cells of the interstitial tissue appear to be undamaged in x-irradiated testes so that one might suppose that the conditioned secondary sexual characters of these sterile mice are normal. The interstitial tissue of the sterile testes is increased in amount relative to the other tissues of the testis as determined by the Chalkley method(37). This observation is accentuated by the closer proximity of the acellular and collapsed tubules. The Chalkley method allows one to utilize the micrometer eye piece, taking each of the 10 figure numbers as points, and the ratio of interstitial to all other types of tissue in the testes can be determined by random sampling. This was done for 25 samples from each testis examined. In the controls this amounted to approximately 15% while in the completely sterile regions of the sterile testes, the interstitial tissue amounted to 44%. This is most clearly illustrated in the sterile testes in which there are clustered tubules containing maturation stages among sterile tubules (Fig. 2 and 4). Eschenbrenner *et al.*(3) found a similar increase in the relative amount of interstitial tissue in testes of adult mice exposed to low dose chronic x-irradiation, but no absolute increase in the interstitial tissue. Whether the cells are all of the same original type (and function) would be difficult to determine, but at least it is all interstitial and not fibrous.

The testes of the x-irradiated but fertile mice can always be recognized in sections by the presence of some tubules completely devoid of maturation stages, generally located at the periphery of the organ. Actual counts showed that the number of sterile over the number of fertile tubules in random centrally located sections of such testes ranged from 9/97 to 52/42 or approximately 9 to 53% sterile tubules. In the latter testes the general impression is of a rather normal organ. The cellular tubules are large and centrally placed while the sterile tubules are very small and located around the periphery, for the most part. One might predict, therefore, the (x-irradiation produced) sterility in these mice will obtain when the number of fertile tubules (*i.e.*, tubules containing recognizable maturation stages) does not go much above 10% and that fertility may be expected from those testes in which something over 40% of the tubules contain maturation figures. Of course, mammalian fertility is somehow related to the production of large numbers of viable spermatozoa, even though the litter may be small, so that a single normal tubule in a testis, producing hundreds of apparently normal spermatozoa, is not sufficient (quantitatively) to insure fertility. This study of the testes casts no light on the matter of specific sensitivity of the various maturation stages because the animal was exposed to x-irradiation during its fetal life when the germ cells were undifferentiated. In most of the sections, even those of sterile mice, there were some tubules containing all maturation stages and this presumes only incomplete inhibition of the normal process of maturation even though a functional number of sperm were not produced.

The ovaries. Size variations in control and x-irradiated ovaries suggest that the sterile ovaries are the smaller, but this would be difficult to quantitate. In the sectioned material the control ovaries at median level showed approximately 14 developing follicles, and 4 to 8 corpora lutea. In contrast, the sterile ovaries of mice x-irradiated *in utero* and examined at 6 months of age contained no follicles of any sort (Fig. 9), and only occasionally small nests of cells that might be arrested follicles (Fig. 15). The sterile ovary consists of a condensed ovarian stroma, interspersed with evidences of fatty degeneration (Fig. 13) and an occasional enlarged cyst (Fig. 10). There is germinal epithelium (Fig. 11), but the usual picture of subepithelial germinal nests is completely abnormal (Fig. 11). In the ovaries of x-irradiated mice which proved to be partially fertile in that they produced fetuses which were de-formed and dead at birth, there were ill-defined and arrested follicles (Fig. 12), some sub-epithelial germinal nests (Fig. 16), and some slight evidences of corpora lutea (Fig. 14). The fertile but x-irradiated ovaries appeared quite normal except for the apparent reduction in the number of developing follicles with ova.

Summary and conclusions. 1. Mice x-irradiated *in utero* to 300 r accumulated in 6 exposures of 50 r each on 6 consecutive days beginning on the 14th day of gestation, reveal various degrees of sterility at 6 months, the period of peak reproductive activity in the mouse. The 10 pregnant females, producing these litters, were all made sterile by this x-irradiation. 2. Within any single litter, there were variations in response of fetuses indicating, possibly, a slight spread in the developmental stage of the gonad primordia at the instant of x-irradiation. Certainly one can hardly visualize a dosimetry sufficiently different within a fetal litter to account for the variations in response. 3. Testes sterility was correlated with the relative number of seminiferous tubules in which maturation stages occur. In those testes where 40% or more of the tubules contain maturation figures, the male was fertile. The completely sterile testes contained 10% or less of tubules having maturation stages. 4. The precursors of the Sertoli cells, interstitial tissue and ovarian stroma are radioresistant. The primordia of the maturation stages and ovarian germinal nests are definitely radiosensitive. It must be emphasized again that at the time of x-irradiation these stages were not yet differentiated so that the sensitivity is related to the primordia of these various cell types, and not to the fully formed cell types. 5. The testes are reduced in size to a degree correlated with the failure of the seminiferous tubules to develop maturation stages. Consequently there is sterility. This failure to develop is accompanied by an apparent relative increase in the interstitial tissue within the testis from some 15% in the controls to 44% in the completely sterile testes. 6. A sub-lethal exposure of the mouse fetus to 300 r will produce sterility in roughly 2/3 of all offspring, with the slightly greater sterility among the males but a greater number of first generation teratologies among the females, mated to control animals. The genetic sequelae of such x-irradiation exposure, while undoubtedly of serious moment, are not considered in this study.

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Lack of Correlation Between Dietary Level of Manganese and Growth Rate of Induced Fibrosarcomas.*† (19635)

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Recently we have demonstrated an effect of the level of dietary manganese on growth in young rats(1). Earlier Maxwell and Bischoff(2) reported that "no effect on tumor growth was noted" in 7 carcinoma-bearing rats that were given intraperitoneal doses of 8-10 mg/kg of Mn $(C_2H_3O_2)_2$ at 3- to 4-day intervals until 45-50 mg Mn/kg had been given. These authors observed, however, that there was less liquefaction of the tumors in the "dosed" animals in the later stages, and that "the occurrence of regressions was also somewhat higher in the dosed series. . . ." Ferguson, Templeton, and Patras(3) reported that "spindle cell sarcoma" transplants grew only about 75% as rapidly in rats on a diet from which the usual "salt-mixture" was omitted. This salt mixture contained small amounts of $MnSO_4$. Popoff (1941)(4) found that minute amounts of Mn, Li, and I_2 added together to the diets of tumor-bearing mice decreased the growth-rate of the tumors to 1/3 or 1/4 of the usual rate. Thus we have one group of investigators(2) reporting "no effect" on tumor-growth from the intraperitoneal administration of extra manganese to tumor bearing rats; a second group (3) reporting a decrease in tumor-growth when the salt-mixture containing manganese is *omitted* from the diet; and a third group (4) reporting that minute amounts of Mn, Li, and I_2 *added* to the diet cause a decrease in the growth-rate of tumors in mice. In all except the first of these experiments, other factors in addition to Mn were varied.

In view of the inconclusive nature of these reports with respect to the growth of tumors, and in view of our demonstration(1) of the effect of the dietary level of Mn on the growth-rate of normal tissue, we have thought it worthwhile to investigate the growth-rate of methylcholanthrene induced-sarcomas of the thigh muscle of the rat with respect to the dietary Mn level.

Experimental. The rats used in these experiments were 30-60-day-old females from a strain that has been inbred at this institution for more than 20 years. In numerous earlier experiments we have shown that the injection of single doses of methylcholanthrene into the posterior muscles of the thigh by our technic produces fibrosarcomas in more than

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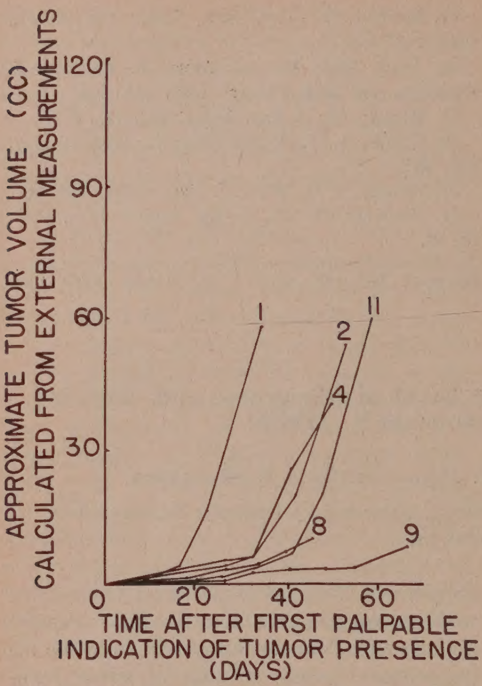

FIG. 1. Rate of growth of tumors in rats receiving no manganese supplement. Figures indicate individual rat numbers.

99% of the animals(5). Twenty rats were divided into groups of 6, 7, and 6. All the animals received a basic diet of Purina Fox Chow and distilled water *ad libitum*. This diet provides about 1 mg of manganese per rat per day.§ Group I (6 rats) received this diet without supplement. Group II (7 rats) received, in addition, 10 mg Mn/rat/day; Group III (6 rats) received 40 mg Mn/rat/day. The Mn supplement, as Mn SO_4, was added to the drinking water. After the rats had been on the respective levels of manganese supplement for 30 days, the tumors were induced by one injection of 3 mg of methylcholanthrene (Eastman) in 0.25 ml of corn oil. The corn oil containing the methylcholanthrene was injected into the posterior muscles of the left midthigh at an intramuscular depth of about 1 cm. Growth of the tumors was estimated by measuring the circumference of the thigh at the level of injection at approximately weekly intervals. From this measurement, and from the assumption that the growing tumors were spheroids, the volume was calculated. For the animals used in these calculations the assumption of spheroid shape was borne out at autopsy.

Results. Results of these experiments are shown in Fig. 1, 2, and 3, which represent graphically the growth of methylcholanthrene-induced tumors in the thigh muscles of rats beginning with the first appearance of palpable growth. The diets contained 1, 11, and 41 mg of manganese per rat per day, respectively. The following comparisons were made between the results in the 3 groups: 1) The time between the injection of methylcholanthrene and appearance of the first palpable tumor, 2) the mean growth-rate from the time of the first appearance of palpable tumor growth to the end of the experiment, and 3) the growth-rate during the 2 weeks' period of maximum growth.

In the first comparison, mean values were so nearly the same that "no significance" was

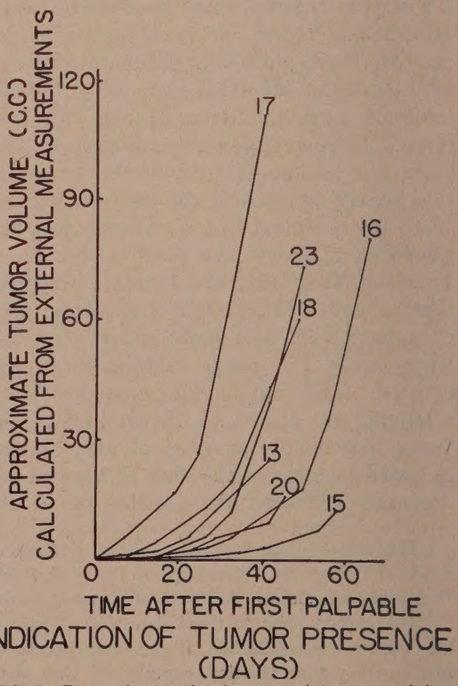

FIG. 2. Rate of growth of tumors in rats receiving 10 mg manganese supplement daily. Figures indicate individual rat numbers.

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§ Purina Assay Charts.

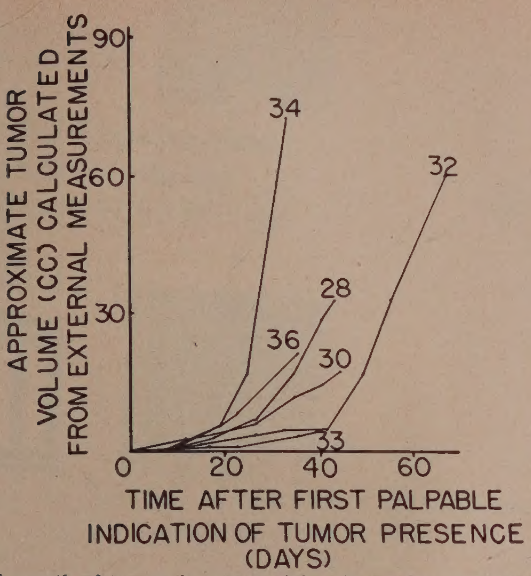

FIG. 3. Rate of growth of tumors in rats receiving 40 mg manganese supplement daily. Figures indicate individual rat numbers.

assumed without subjecting the data to statistical analysis. In the second and third, tests of significance of difference between means were applied, with correction for sample size. In every case the difference between means showed a value of "P" which ranged from 0.40 to 0.30 as a maximum and from 0.20 to 0.10 as a minimum.

Inasmuch as a tumor has been growing for an unknown time before it is first palpable, initial palpability as a measure of initial tumor-growth involves an unknown degree of error in the time factor. It is possible that if timing could be accomplished with greater precision, significant differences in the rate of tumor-growth in rats on different dietary levels of manganese might appear, both in the time between injection of methylcholanthrene and initial tumor-growth, and in the mean growth-rate of tumors. However, timing of initial growth does not affect the third comparison, the maximum growth-rate of the tumor.

From these experiments it must be concluded that, with the technics at our disposal, at dietary levels of manganese of 1, 11, and 41 mg per rat per day, the level of manganese intake has not been shown to influence the growth-rate of methylcholanthrene-induced sarcomas.

No difference in the degree of "liquefaction" of tumors was observed between the different groups, and no regressions occurred in any of the tumors.

Conclusions. At dietary levels of manganese of 1, 11, and 41 mg/rat/day, there is an apparent lack of correlation between level of dietary manganese intake and growth rate of methylcholanthrene-induced sarcomas of the thigh muscles of white rats.

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